THE MOLECULAR EFFECTS OF THE ANTITUMOR ANTIBIOTIC CORDYCEPIN IN *SACCHAROMYCES CEREVISIAE*

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II Abbreviations

ADA	adenosine deaminase
ATP	adenosine triphosphate
bp	base pair
cAMP	cyclic adenosine monophosphate
CoDP	cordycepin diphosphate
CoMP	cordycepin monophosphate
CoTP	cordycepin triphosphate
CTD	C-terminal Domain
CTP	cytidine triphosphate
CUT	cryptic unstable transcript
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
GO	gene ontology
GTP	guanosine triphosphate
hnRNA	heterologous nuclear RNA
mRNA	messenger RNA
NPC	nuclear pore complex
nt	nucleotide
NTP	nucleotide triphosphate
OD_{600}	optical density at λ = 600 nm
PAP	poly(A) polymerase
PIC	pre-initiation complex
RNA	ribonucleic acid
rRNA	ribosomal RNA
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
RNP	ribonucleoprotein

TdT	termina deoxynucleotidyl transferase
TEC	transcritpin elongation complex
tRNA	transfer RNA
UTP	uridine triphosphate

III Summary

Cordycepin is an analogue of the nucleoside adenosine and displays various biological activities, including anti-microbial, anti-fungal and anti-proliferative. The absence of a 3' hydroxyl group leads to termination of RNA synthesis when this drug is incorporated into a RNA strand during transcription or pre-mRNA polyadenylation. The aim of this thesis was to gain further insight into the molecular mode of action of cordycepin in the budding yeast *Saccharomyces cerevisiae* and to identify new factors involved in RNA synthesis pathways. Initially, we analysed the molecular effects of cordycepin on RNA metabolism in wild-type strains. Consistent with the proposed function as a RNA chain terminator we found that cordycepin caused a general decrease of mRNA levels. However, we also identified additional, unexpected effects of the drug: dramatic changes of mRNA 3' ends, which likely occurred independently from the termination of the polyadenylation reaction, and furthermore, the appearance of elongated transcripts. Most interestingly, we provide evidence that cordycepin incorporation into poly(A) tails of unstable transcripts may inhibit RNA surveillance by the TRAMP-exosome pathway leading to a stabilisation of the respective RNAs. In an attempt to identify all cellular pathways that are targeted by cordycepin we performed systematic and genome-wide screening. This identified genes that affected the ratio of ATP/CoTP and genes that stabilise mRNAs. Our analyses suggest that the relative levels of these nucleotide triphosphates determine cordycepin toxicity and that inhibition of RNA synthesis was the primary cause for growth inhibition. More unexpectedly, we identified the SWR1 chromatin remodelling complex and its substrate, the histone H2A variant Htz1p, which could be linked to RNA surveillance activities in our analyses. Further functional tests revealed that many of the identified genes exhibited aberrant global poly(A) tail length distribution and genetic interactions with the 3' end factor *RNA14* pointing to a potential role in pre-mRNA synthesis and processing. Finally, we established that cordycepin sensitive growth linked elevated levels of polyphosphate to the inhibition of poly(A) polymerase, which could indicate a regulatory role for polyphosphate in gene expression in yeast. This work establishes novel physiological roles for cordycepin and identifies new targets and pathways that mediate the effect of the drug. Thus, our results may be helpful to further understand and exploit the therapeutic potential of cordycepin on the one hand and to connect the cellular targets of cordycepin to mRNA synthesis and pre-mRNA processing pathways on the other hand.

IV Zusammenfassung

Cordycepin ist ein Adenosinderivat, welches diverse biologische Aktivitäten aufweist, einschliesslich einer Hemmwirkung gegen Mikroben, Pilze und Zellproliferation. Das Fehlen der 3' Hydoxylgruppe führt zur Termination der RNA Synthese, sobald Cordycepin während der Transkription oder der Polyadenylierung der prä-mRNA in den RNA Strang eingebaut wird. Das Ziel dieser Arbeit war, den genauen Wirkungsmechanismus von Cordycepin in der Hefe *Saccharomyces cerevisiae* zu analysieren und dabei bisher unbekannte Faktoren zu identifizieren, die in den Prozess der RNA Synthese involviert sind. In einem ersten Schritt untersuchten wir die molekularen Effekte von Cordycepin auf den RNA Metabolismus im Wildtyp. Das Adenosinderivat verursachte eine generelle Abnahme der RNA Level. Dies ist konsistent mit der angenommen Wirkung von Cordycepin, dass die RNA Synthese terminiert wird. Darüber hinaus identifizierten wir unerwartete Effekte von Cordycepin: zum einen starke Veränderungen am 3' Ende der mRNA, welche sehr wahrscheinlich unabhängig vom Abbruch der Polyadenylierung zustande kommen. Zum anderen das Auftreten von verlängerten RNA Transkripten. Die Analysen ergaben ausserdem, dass der Einbau von Cordycepin in den Poly(A) Schwanz von instabilen Transkripten den RNA Überwachungsmechansimus inhibieren könnte, der durch den TRAMP-Komplex und das Exosom gesteuert wird, was zu einer Stabilisierung von diesen RNA Transkripten führt. In einem Versuch, alle zellulären Prozesse, die von Cordycepin attackiert werden, zu identifizieren, haben wir einen systematischen, genomweiten Screen durchgeführt. Wir identifizierten Gene, die das Verhältnis von ATP zu CoTP beeinflussen und Gene, welche mRNA stabilisieren. Unsere Ergebnisse deuten darauf hin, dass die relativen Mengen von beiden Nukleotidtriphosphaten die Toxizität von Cordycepin bestimmen und dass die Inhibierung der RNA Synthese die Hauptursache für die Wachstumshemmung ist. Zudem identifizierten wir den SWR1 Komplex und sein Substrat Htz1p, eine Histonvariante von H2A. Beide wurden durch unsere Ergebnisse mit dem RNA Überwachungsmechanismus in Verbindung gebracht. Funktionelle Tests ergaben, dass viele der identifizierten Gene eine anormale Längenverteilung der Poly(A) Schwänze aufwiesen. Gleichzeitig interagieren viele Gene genetisch mit *RNA14*, einem Faktor, der an der Bildung des 3' Endes der RNA beteiligt ist. Beide Beobachtungen lassen darauf schliessen, dass diese Gene eine Funktion innerhalb der prä-mRNA Synthese und deren Prozessierung aufweisen. Schliesslich haben wir festgestellt, dass sensitives

Wachstum in Anwesenheit von Cordycepin eine erhöhte Polyphosphatkonzentration an einer Inhibierung von Poly(A)polymerase koppelt. Dies könnte auf eine Funktion von Polyphosphat in der Regulation von Genexpression in Hefe hindeuten. Die vorliegende Arbeit postuliert neue physiologische Funktionen von Cordycepin und identifiziert neue Gene oder Prozesse, welche die Wirkung von Cordycepin herbeiführen. Unsere Resultate könnten deshalb dazu beitragen, das therapeutische Potential von Cordycepin weiter auszuschöpfen und die biologische Wirkung von Cordycepin mit der mRNA Synthese und der prä-mRNA Prozessierung in Verbindung zu bringen.

V Introduction

1 Cordycepin

1.1 The genus of the fungus *Cordyceps*

Cordycepin is a compound which was first isolated from the culture media of the fungus *Cordyceps militaris* (Cunningham et al., 1950). *Cordyceps* is a genus of ascomycete fungi, which consists of around 400 species. All of them are parasites of insects or fungi and often exhibit a high degree of host specificity. When a *Cordyceps* attacks a host, the mycelium invades the host tissue and eventually replaces it. The fungus ruptures the host body and forms the sexual perothecial stroma that is connected to the dead larva and which grows upward to emerge above the soil surface (figure 1). What exactly kills the host is not clear, however, it might be the accumulation of the biomass or the toxins produced by the fungus. Several *Cordyceps* species are producing cordycepin, but the best known species of the genus is *Cordyceps sinensis*, because it is one of the most famous traditional Chinese medicines. The complex of the fungus and the larva body which is called Dong Chong Xia Cao (winter worm summer grass) has been used as a health food and tranditional medicine in China for hundreds of years. Now it is available in different forms: crude extracts, water, ethanol, methanol and ethyl acetate extracts, pure compounds or whole fungi (with or without the dead hosts). It has been assigned to have various beneficial effects on humans: anti-aging, pro-sexual, anti-cancer, anti-metastatic, anti-oxidant, anti-inflammatory, insecticidal, anti-microbial, and immune boosting; it helps against respiratory, renal, liver, neural and cardiovascular diseases. For most of these effects, the scientific evidences are rather poor (Paterson, 2008).

The fungus is endemic to the alpine habitats of the Tibetan Plateau above 3000 m in south-western China, and there has been large-scale harvesting of the wild material. The fungus has officially been classified as an endangered species. The price of natural products of *C. sinensis* is over 12000 US $/kg and the market is increasing (Paterson, 2008). As a consequence, living strains have been isolated from natural *Cordyceps* and cultivated in large quantity by bioreactor technology, which is a promising method to encounter the needs of human consumption and to reduce the pressure on natural resources

of the species. *In vitro* culture of the fungus has been employed increasingly and it is generally accepted that cultivated *C. sinensis* possesses the same benefits and properties as *C. sinensis* natural herbs.

Various bioactive components in *Cordyceps* species have been reported. These include cordycepin and other adenosine derivatives, ophicordin (antifungal agent), L-tryptophan, polysaccharides and ergosterol (Wu et al., 2005). *C. sinensis* first gained worldwide attention when it was revealed that several Chinese runners who broke world records in 1993 had included this fungus in their diet as part of their training program (Paterson, 2008).

Especially Chinese research groups have been investigating the various effects of *Cordyceps* on the human body also in recent studies (Zhu et al., 1998; Li and Tsim, 2004). Solid or liquid fermentation has been widely used for the production of *Cordyceps* mycelial body mass and components and therefore, there has been an increasing number of studies on the liquid fermentation of *C. sinensis* or some other valuable *Cordyceps* species (Xiao et al., 2004; Kim and Yun, 2005; Mao et al., 2005). Most of these studies try to optimise nutrients and conditions for mycelial growth and the yield of cordycepin production. Ammonium feeding (5 – 10 mmol/l) to the mycelial culture of *C. sinensis* has been shown to enhance the intracellular cordycepin accumulation and the exopolysaccharide production (Leung and Wu, 2007).

Figure 1 *Cordyceps* species on insect hosts. (Paterson, 2008)

1.2 Chemical structure of cordycepin

Cordycepin, or 3' deoxyadenosine, is a derivative of the nucleoside adenosine differing from the latter by the absence of the OH group in the 3' position of its ribose entity (figure 2). It was first isolated by Cunningham et al. (1950) from the liquid medium of the fungus *Cordyceps militaris* (Cunningham et al., 1950). The mold was co-cultured with the bacteria *Bacillus subtilis* (*B. subtilis*) and found to inhibit the growth of the bacterium after 15 days slightly, and after 24 days to a more pronounced degree. A crystalline compound was isolated from the culture filtrates, which appeared to be responsible for the observed inhibitory effect. This compound was named 'cordycepin'. Surprisingly, it had no inhibitory effect on the growth of other prokaryotes as for example *Staphylococcus aureus* and *Escherichia coli*. Initially, a wrong structure was assigned to cordycepin (Bentley et al., 1951). The authors concluded from physical properties of the compound and the product of different chemical reactions that the sugar moiety of cordycepin was a 3-D-deoxyapiose, a branched-carbon chain 3' deoxypentose, which was named cordycepose (figure 2). The branch-chain CH_2OH was assigned to C-4' instead of C-5' of the furanose ring resulting in the wrong sugar moiety. Additional evidence substantiating cordycepose as a branched-chain 3-deoxypentose was presented 1955 (Raphael and Roxburgh, 1955). The authors reported the total chemical synthesis of cordycepose and its conversion to the crystalline p-nitrophenylosazone. The osazone of the synthetic branched-chain sugar had the same melting point as that one obtained from the cordycepose isolated from the metabolite cordycepin. A mixture of them showed no depression in melting point, which was considered additional proof that the structure assigned to the carbohydrate moiety of cordycepin was indeed a branched-chain sugar.

The first indication that the sugar moiety of cordycepin was not a branched-chain sugar was reported in 1963 (Klenow, 1963a). When cordycepin isolated from culture filtrates of *C. militaris* was added to cultured Ehrlich ascites tumor cells, three additional major peaks appeared in the elution profile of an anion-exchange chromatography of the acid-soluble fraction. These three peaks had very similar chemical properties to nucleotides of the base adenine and could be identified as cordycepin mono-, di-, and triphosphate. The monophosphate of cordycepin was dephosphorylated in presence of snake venom 5' nucleosidase with the same rate than an equimolar amount of 2' dAMP. Assuming that the structure of cordycepin was as proposed (Bentley et al. 1951) and therefore does not

contain a 5' hydroxyl group, Klenow's result indicated that the specificity of snake venom 5' nucleotidase included the hydrolysis of phosphate of branched-chain sugars with hydroxymethyl groups. This seemed to be highly unlikely. Subsequent physical and chemical studies (Kaczka et al., 1964b; Suhadolnik and Cory, 1964; Hanessian et al., 1966) have shown that the structure of the carbohydrate moiety of cordycepin as proposed by Bentley et al. (1951) was incorrect and that cordycepin is a 3' deoxynucleoside.

The isolation of cordycepin from culture filtrates of *Cordyceps militaris* has been simplified and the yield has been improved over the years (Kredich and Guarano, 1960; Kaczka et al., 1964a; Frederiksen et al., 1965; Chassy and Suhdolnik, 1969), the latter of those reports was regarded as a standard extraction method for many years, until the chemical synthesis of cordycepin replaced the extraction procedure.

1.3 Biochemical properties of cordycepin

The biochemical properties of cordycepin were of high interest. Intriguing was particularly the ovservation, that cordycepin inhibited the growth of *B. subtilis* but not of other bacteria. In the early 60s it was suggested that cordycepin might inhibit the *de novo* purine

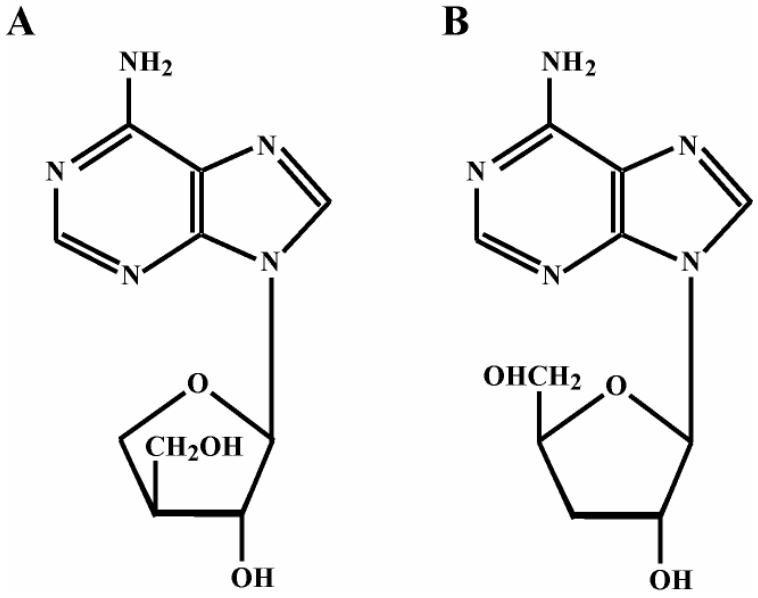

Figure 2 Chemical structures assigned to cordycepin.

***A** Initially proposed chemical structure with the sugar moiety cordycepose (Bentley et al., 1951). **B** Correct structure of cordycepin as a derivative of adenosine.*

biosynthesis in *B. subtilis* (Rottman and Guarino, 1964a). In the presence of cordycepin, less phosphate is incorporated into RNA and DNA, and only the additional supply of adenine or guanine can effectively reverse this effect (Rottman and Guarino, 1964b). In a subsequent study, it was shown that cordycepin is phosphorylated to cordycepin monophosphate in *B. subtilis* and that this form of the nucleotide inhibited the activity of the enzyme phosphoribosyl pyrophosphatate amidotransferase (Rottman and Guarino, 1964c).

A possible anticancer activity of cordycepin was first described in Ehrlich ascites cancer cells. Cordycepin increases the survival time of mice bearing the Ehrlich ascites tumor (Jagger et al., 1961). When tumor cells and cordycepin were administered at the same time, followed by daily injections of cordycepin for 7 days (15 – 200 mg/kg body weight), a significant increase in survival time of the mice was observed. Even when the tumor has been allowed to develop 5 days prior to cordycepin treatment starts, the animals survived significantly longer than control animals (Jagger et al., 1961). Trying to find the biochemical mechanism for this anti-tumor effect of cordycepin, the nucleotide distribution was analysed in the tumor cells. This study revealed that these cells were able to phosphorylate cordycepin to the mono-, di-, and triphsophates in a very efficient process (Klenow, 1963a). 80% of the added cordycepin was converted into either mono-, di-, or triphosphates within 30 minutes (Klenow, 1963b). Besides a first strong evidence for emending the sugar moiety of cordycepin to a 3' deoxypentose, this important observation also unveiled the first biochemical property of cordycepin in the cell: its ability to become part of the nucleotide pool. Follow-up studies revealed a concentration dependent effect of cordycepin: low concentrations (0.5 – 1.0 µmol/ml) of cordycepin resulted in the accumulation of cordycepin-triphosphate (CoTP) in the cells and did not inhibit the incorporation of phosphate into DNA, whereas higher concentrations (2 µmol/ml and higher) led also to the formation of cordycepin monophosphate (CoMP) and –diphosphate (CoDP), which is accompanied by a rapid decrease of nucleotide levels in the cells followed by a pronounced inhibition of incorporation of phosphate into nucleic acids (Klenow, 1963b). Both effects could be prevented by simultaneous addition of adenosine, but not 2' deoxyadenosine (Klenow, 1963b). This lead to the conclusion, that the formation of CoMP and CoDP were the more immediate inhibitory substances rather than CoTP. This theory was supported by results with experiments using cordycepin-*N*-oxide which is slowly reduced to cordycepin in suspension with tumor cells (Frederiksen and Klenow,

1962). RNA synthesis was blocked after 1.5 hours, DNA synthesis after 3 hours (Frederiksen, 1963). It was concluded that CoDP probably causes the inhibition of DNA synthesis and CoTP is responsible for RNA synthesis inhibition (Frederiksen, 1963).

In a subsequent report it was shown that under experimental conditions in which CoTP accumulated there is no significant inhibition in the incorporation of phosphate into the DNA of the tumor cells, but a strong inhibition in the incorporation of adenine into DNA (Klenow and Overgaard-Hansen, 1964). These findings led to the assumption that CoTP does not inhibit the process of DNA synthesis, but rather a step in the metabolic pathway required for the conversion of adenine into adenosine (Klenow and Overgaard-Hansen, 1964). In subsequent analysis, CoTP was added to the supernatant of tumor cells and it could be demonstrated that the reaction ATP + ribose-5-phosphate → ribosephosphate pyrophosphate (PRPP) + AMP catalysed by the enzyme ribosephosphate pyrophosphokinase is inhibited (Overgaard-Hansen, 1964). Since PRPP is a key enzymatic activity required for growth and is also an obligatory reactant in the *de novo* synthesis of purines, pyrimidines and pyrimidine nucleotides, it is reasonable to assume that the inhibition caused by cordycepin in Ehrlich ascites tumor cells may be partially attributed to the inhibition that CoTP exerted on the synthesis of PRPP (Overgaard-Hansen, 1964). This proposed inhibition model was very similar to the model for the bacteria *B. subtilis* (Rottman and Guarino, 1964c).

However, the inhibition of RNA synthesis came in focus as the main target effect of cordycepin. The effect of CoTP on RNA polymerase from tumor cells was studied and in an enzyme assay, addition of CoTP almost completely prevented incorporation of AMP into RNA, but radio-labelled phosphate incorporation in DNA could not be detected (Klenow and Frederiksen, 1964a). This observation was subsequently followed up by *in vivo* data. Under conditions in which CoTP accumulated in tumor cells, the incorporation of phosphate into RNA was inhibited by 50% after 60 minutes (Klenow and Frederikson, 1964b). This inhibitory effect of cordycepin did not occur uniformly: the cytoplasmic and the nucleochromosomal RNA fractions were inhibited profoundly, whereas the nucleoplasmic RNA fraction did not display any inhibition (Klenow and Frederikson, 1964b).

Incorporation of formate, glycine, hypoxanthine, adenine and guanine into purine RNA-precursors was studied in ascites tumor cells. In presence of cordycepin the incorporation of these five compounds into purine RNA-precursors was inhibited more than 80% (Shigeura and Gordon, 1965). Since the incorporation of a *de novo* purine

precursor or intact purine into RNA is inhibited, and not the formation of the precursor, it appears that the RNA polymerase might be inhibited (Shigeura and Gordon, 1965). This was demonstrated by enzyme assays with RNA polymerase isolated from *Micrococcus lysodeikticus*. CoTP inhibits the polymerisation reaction, whereas CoMP and CoDP do not have an effect (Shigeura and Gordon, 1965). CoTP inhibits, besides the synthesis of RNA also poly(A) formation, but does not inhibit the formation of poly(U) or DNA.

Cordycepin addition also revealed a cytotoxic effect on human tumor cells (H. Ep. No. 1) in culture (Rich et al., 1965). Adenosine administration prevented growth inhibition, but could not reverse the inhibition once it has occurred. It was suggested that adenosine competes with cordycepin for phosphorylation, however, marked effects of cordycepin on RNA, DNA or protein content *in vivo,* or the incorporation of cordycepin into RNA or DNA could not be demonstrated. However, an opposing result was published in the same year demonstrating that cordycepin is readily phosphorylated into CoTP and incorporated into RNA and DNA of the human cancer cells, presumably at the 3' termini (Cory et al., 1965). These results together with other studies (Shigeura et al., 1966) led to the model that cordycepin is taken up by the cells, phosphorlyated to CoTP and appears to inhibit RNA synthesis by incorporation into the terminal position of RNA. Thereby it may block further growth of the polynucleotide chain because of the lacking 3' OH group. CoTP can be inserted into a growing RNA chain by the normal 5'-3' phosphodiester bond, but the next 5'-3' bond can not be formed. Cordycepin was specified as a RNA chain terminator, an activity that was previously demonstrated for the adenosine derivative 2',3' dideoxyribonucleotide and other nucleotide derivatives lacking the 3' OH group (Atkinson et al., 1969).

None of the phosphorylated cordycepin nucleotides acted as a substrate for the enzyme ribonucleotide reductase isolated from *Lactobacillus leichmannii* and *Escherichia coli* (*E. coli*) indicating that Cordycepin is not converted to 2',3' dideoxycordycepin (Chassy and Suhadolnik, 1968). In HeLa cells, in was observed that cordycepin inhibited total RNA synthesis by 50% already at a concentration of 25 µg/ml (Siev et al., 1969). DNA synthesis, in contrast, remained unaffected even at higher concentrations. This insensitivity of DNA and protein synthesis showed that cordycepin did not directly interfere with general cell metabolism and that the reported inhibition of DNA synthesis (Frederiksen, 1963) probably via inhibition of purine synthesis (Overgaard-Hansen, 1964). This probably is not the primary inhibitory effect of CoTP, but may be a secondary consequence of the inhibition of RNA synthesis.

The insensitivity of *C. militaris* against its own cordycepin has also interested scientists. It could be shown, that cordycepin was not taken up by *C. militaris,* neither in the early stages of growth nor at the time of cordycepin production (Chassy and Suhadolnik, 1969). Apparently, once cordycepin is excreted into the medium, it is not taken up by the cell again, which is probably a mechanism of self-protection. In the case of *E. coli*, it has been shown that this bacterium lacks an endogenous adenosine kinase activity (Neuhard and Nygaard, 1987), which explains the insensitivity against cordycepin.

1.4 Effect on RNA polymerases and poly(A) polymerases

In the year 1965, it was reported for the first time in *in vitro* experiments, that CoTP is incorporated into RNA by partially purified RNA polymerase from *M. lysodeikticus* (Shigeura and Gordon, 1965) and from this time on, it has been generally accepted that the main mode of action of cordycepin is its inhibitory effect on RNA polymerases. The open question was which polymerase showed the strongest inhibition towards CoTP and this question has still not been answered completely until today.

In HeLa cells the addition of cordycepin affected both the amount and size distribution of nucleolar RNA, whereas nucleoplasmic RNA seemed unaffected in size and amount (Siev et al., 1969). The amount of labelled 18S RNA decreased to 25% of control, the production of 28 S RNA was completely suppressed and the formation of incomplete 45S RNA could be detected. This led to the suggestion that 18S RNA is located close to the 5' end of the 45S precursor RNA, and could also be produced from incomplete precursor molecules. 28S RNA must be located near the 3' end and the complete synthesis of the precursor is a prerequisite for 28S RNA production.

In contrast to these observations, Penman and co-workers showed that cordycepin mainly affected mRNA production leading to the conclusion that cordycepin might have 3 different effects on RNA synthesis (Penman et al., 1970): The first is complete inhibition, which is characteristic for the synthesis of mRNA (Penman et al., 1970). The second effect is premature termination of nascent RNA and release of the defective RNA chain and occurs in the nucleolus (Siev et al., 1969). A third response is insensitivity, which was assigned to the synthesis of nuclear heterogeneous RNA.

A decade later, *in vitro* studies with purified RNA polymerases from HeLa cells and *E. coli* showed that CoTP inhibited the activity of eukaryotic and prokaryotic polymerases to the same extent (Maale et al., 1975), although cordycepin did only inhibit growth of HeLa cells, but not that of *E. coli.* The poly(A)-synthesising enzymes purified from HeLa cells and *E. coli* in this study were not more sensitive to CoTP than to the naturally occurring 2' deoxyadenosine (Maale et al., 1975).

In 1976, kinetic constants for CoTP were published for the first time with the RNA polymerases from the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) (table 1) (Horowitz et al., 1976). According to the calculated kinetic constants, the inhibition of effect of CoTP towards the different RNA polymerases *in vitro* differed significantly. The inhibitory effect of CoTP on RNA polymerase II was the highest. Inhibition of poly(A) polymerase was comparable with that observed with RNA polymerase III and interestingly, RNA polymerase I was completely insensitive to CoTP. This result suggested that at least in yeast, polyadenylation might not be the major target of CoTP and that cordycepin affected the polymerases to a different extent. These findings were not consistent with previously presented suggestions (Siev et al., 1969), although *in vivo* observations in yeast have not been carried out yet.

enzyme	K_m (μM) ATP	K_i (μM) CoTP
RNA polymerase I	32	-
RNA polymerase II	12	0.3
RNA polymerase III	32	3.0
poly(A) polymerase	56	4.6

Table 1 Calculated kinetic constants with enzymes purified from *S. cerevisiae* (Horowitz et al., 1976)**.**

One year later, kinetic parameters for mammalian enzymes were presented (Müller et al., 1977). In this study, the effect of CoTP on isolated DNA polymerases was also tested. Mammalian α and β DNA polymerases were both not affected by CoTP underscoring the model that the inhibition of DNA synthesis was not the primary effect of CoTP. Mammalian RNA polymerases (table 2) were selectively blocked as was observed with the RNA polymerases from *S. cerevisiae* (Horowitz et al., 1976), but the inhibitory

constants were not comparable to the constants determined for the yeast polymerases. Since the inhibition of all enzymes was competitive with respect to ATP, the authors suggested using the K_i/K_m value, which gave insight into the inhibition relative to their respective affinities. The lower the K_i/K_m value is the stronger is also the inhibitory potency of a substance. The three different RNA polymerases were moderately inhibited by CoTP. The inhibitory effect of CoTP was higher towards RNA polymerase II (ratio 1.67) than towards RNA polymerase I and III (ratios 2.52 and 2.71). The potency of CoTP to inhibit poly(A) polymerase or terminal riboadenylate transferase was significantly higher. Moreover, in enzyme studies with CoTP and poly(A) polymerase, the resulting product oligo(pA)6-CoMP had no more initiator capacity for the enzyme, substantiating that the incorporated CoMP acted as chain terminator (Müller et al., 1977).

enzyme	K_m (µM) ATP	K_i (µM) CoTP	K_i/K_m
RNA polymerase I	16.8	42.3	2.52
RNA polymerase II	23.9	39.9	1.67
RNA polymerase III	27.1	73.4	2.71
poly(A) polymerase	34.7	7.3	0.21
terminal ribo-adenylate transferase	21	16	0.75

Table 2 Influence of CoTP on RNA polymerases, poly(A) polymerase and terminal riboadenlyate transferase (Müller et al., 1977).

The authors tried to show the same inhibition pattern also in an *in vivo* situation. But the used concentration of cordycepin produced different consequences. At a low concentration (< 1 µM) of the compound cell proliferation was not influenced, although cordycepin was incorporated into RNA. Higher concentrations (5 µM) caused a drastic reduction of the amount of polysomes, indicating a strong influence of cordycepin on the availability of mRNA in the cytoplasm (Müller et al., 1977).

In contrast to the proposed kinetic constants, Rose and co-workers published in the same year, that 60 µg/ml CoTP caused an inhibition of RNA polymerase II activity isolated from rat liver of only 5%, whereas RNA polymerase I activity was inhibited by 70% (Rose et al., 1977). However, poly (A) polymerase was inhibited most although the

"chromatin associated" poly(A) polymerase was around 30 times more sensitive to CoTP than is the "free" poly(A) polymerase. The presented results were contradictory to the kinetic parameters presented in the same year (Müller et al., 1977), but were consistent with the observed *in vivo* findings (Siev et al., 1969).

Further *in vitro* studies with isolated rat liver nuclei revealed that CoTP was capable to significantly inhibit also the synthesis of 5S RNA and tRNA at concentrations between 50 µg/ml and 200 µg/ml (Leonard and Jacob, 1979), which has previously been suggested at least for 5S RNA (Müller et al., 1977). This result clearly demonstrated that all three RNA polymerases could be inhibited by the ATP analogue and that none of them is insensitive as proposed previously (Horowitz et al., 1976).

CoTP inhibits also RNA polymerases I and II isolated from the slime mold *Distyostelium discoideum* in a competitive manner to ATP, although inhibition towards polymerase I is a bit stronger (Saneyoshi et al., 1981). The Michaelis constant K_m for RNA polymerase II and CoTP was estimated to be 5.6 µM and the inhibition constant K_i 0.8 µM. Similar inhibition values were calculated for 3' dUTP and 3' dCTP, both of which are thought to act as chain terminators for RNA polymerases. Towards RNA polymerases I and II isolated from cherry salmon liver, CoTP had almost the same inhibitory effects (Nakayama and Saneyoshi, 1985). Analysis with RNA polymerase and poly(A) polymerase from vaccina virus indicate that poly(A) polymerase is much more sensitive towards CoTP than RNA polymerase (Shuman and Moss, 1987), but *in vivo* studies of the effect of CoTP have not been presented.

In a newer study presenting also a new method for kinetic parameter determination, the K_m and K_i constants of ATP and CoTP were determined for influenza and yeast poly(A) polymerase (table 3) (Hooker et al., 2001). For the yeast poly(A) polymerase, the K_i value for CoTP was significantly lower than the K_m value for ATP resulting in significant inhibition of yeast poly(A) polymerase at relatively low concentrations of CoTP, whereas the K_i value determined for inhibition by CoTP was similar to the K_m value for ATP with influenza polymerase According to their measurements, the poly(A) polymerases from different species do not show the same sensitivity towards CoTP and influenza poly(A) polymerase seems to be surprisingly resistant to CoTP (Hooker et al., 2001). Similar low K_i values for CoTP have been previously found with chromatin associated poly(A) polymerase from rat liver and poly(A) polymerase from yeast (Horowitz et al., 1976; Rose et al., 1977).

enzyme	K_m (µM) ATP	K_i (µM) CoTP	K_i/K_m
yeast poly(A) polymerase	50	0.6	0.012
influenza poly(A) polymerase	186	117	0.629

Table 3 Kinetic constants for poly(A) polymerases from yeast and influenza virus (Hooker et al., 2001)**.**

A different inhibition mechanism for CoTP was proposed in 1978. It was found that CoTP is not incorporated into poly(A) RNA by poly(A) polymerase in *vitro* and therefore did not act as a chain terminator as previously reported (Müller et al., 1977; Horowitz et al., 1976), but rather inhibited poly(A) synthesis by competing with ATP for the active binding site (Koch and Niessing, 1978). In an extensive study with yeast poly(A) polymerase and various different nucleotides, it could be demonstrated that poly(A) polymerase indeed incorporates CoTP into an RNA primer (Martin and Keller, 1998). The RNA primer was elongated only by a single CoTP indicating that cordycepin acts as an RNA chain terminator. CoTP was transferred very efficienty by poly(A) polymerase, in the enzyme assay 100% of the RNA primers were elongated by CoTP. Dideoxynucleotides, which also terminate RNA chain elongation, were incorporated less efficiently. These results suggested using CoTP and poly(A) polymerase for labelling 3' ends of RNA *in vitro*, for example in poly(A) tail length analysis.

In summary, it can be concluded that CoTP highly inhibits the activity of poly(A)polymerase as most of the studies clearly demonstrate the highest inhibition on this enzyme, but surprisingly not in every organisms. The results of the RNA polymerases I, II and III are contradictory and moreover, the *in vivo* observations of different RNA levels are as well not consistent. In the case of yeast, CoTP showed the strongest inhibitory potency on RNA Pol II and poly(A) polymerase was only moderately inhibited (Horowitz et al., 1976). However, a newer study exposed CoTP as a potent inhibitor of the yeast poly(A) polymerase (Hooker et al., 2001). Newer data on yeast RNA polymerases are unfortunately not available.

1.5 Biological activities of cordycepin

Cordycepin is a nucleoside with a broad spectrum of biological activities, which are described in the following chapters in more detail.

The effectiveness of cordycepin in mammals is limited by its rapid deamination to the inactive metabolite 3' deoxyinosine (figure 3). The reaction is catalysed by adenosine deaminase (ADA), an abundant and highly active enzyme present in many tissues and in the blood (Agarwal et al., 1975). Co-administration of coformycin (CF) or 2' deoxycoformycin (dCF), both of which are potent inhibitors of adenosine deaminase (Agarwal and Parks, 1977) (figure 4), markedly increased the efficacy of cordycepin both *in vitro* and *in vivo* (Adamson et al., 1977). In cancer patients, deoxycoformycin (as pentostatin) is active in the treatment of prolymphocytic and hairy cell leukaemia, even for those patients with prior chemotherapy (Döhner et al., 1993; Ho et al., 1990).

Although the combination of cordycepin with an ADA inhibitor can improve the bioactivity of cordycepin, scientists are still trying to find alternatives to avoid the co-administration of an ADA-inhibitor. Aminoacyl derivatives of nucleotides are stable in neutral and alkaline conditions and might be used as effective prodrugs (Guo, 1994).

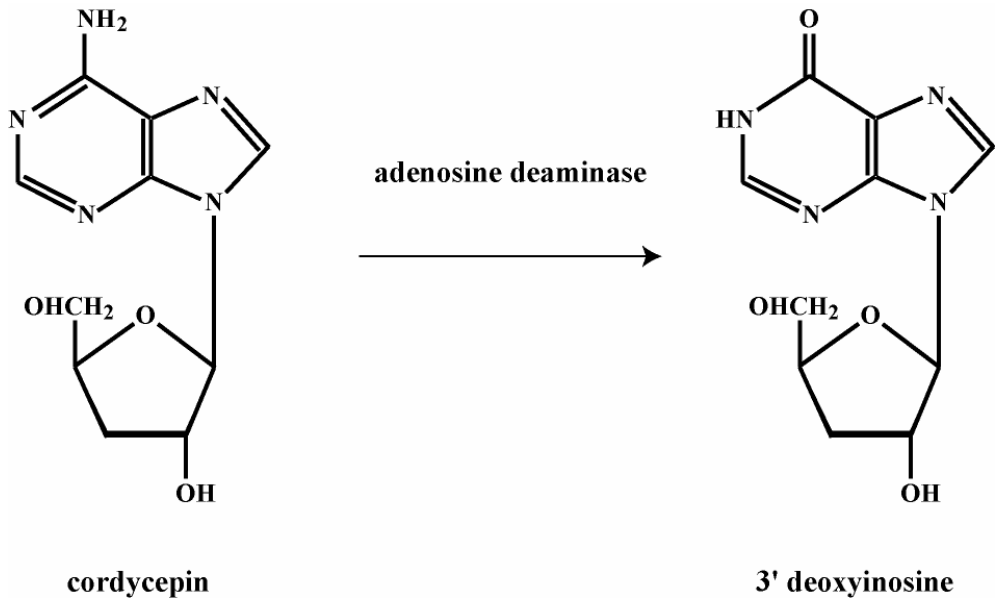

Figure 3 Reaction catalysed by adenosine deaminase.

The amino group at N7 of the purine base is deaminated to oxygen by the enzyme adenosine deaminase resulting in 3' deoxyinosine and ammonium.

Cordycepin was chemically changed to N-alkyl-cordycepin, N-propionyl-cordycepin, N-octanoyl cordycepin N-lauroyl-cordycepin and N-stearoyl-cordycepin, and orally administrated to mice (Wei et al., 2008). With this approach, the primary amine of adenine is protected from oxidation. Cordycepin levels measured in blood revealed that all derivatives can be transformed to cordycepin *in vivo* and that the measured half-time of cordycepin levels in blood highly increases (Wei et al., 2008).

A different method to protect cordycepin from deamination is the development of a cordycepin-layered double hydroxides (LDHs) [Mg-Al-cordycepin] nanohybrid (Yang et al., 2006). The incorporation of organic guests into LDHs has received much attention recently because of the potential uses of the resulting inorganic-organic nanohybrid materials in different fields such as optics, catalysis, nanocomposite engineering and medical science (Khan and O'Hare, 2002). The [Mg-Al-cordycepin] nanohybrid is engulfed by cells by endocytosis. In the lysosome, the LDHs layers are dissolved slowly by the low pH-value and the interlayer biomolecule can be released inside the cell. The [Mg-Al-cordycepin] nanohybrid was tested with human leukaemia U937 cells and found to inhibit the growth of these cells more efficiently than cordycepin addition alone (Yang et al., 2006). This result suggests that LDHs could be a promising nanocarrier for cordycepin delivery into the cells.

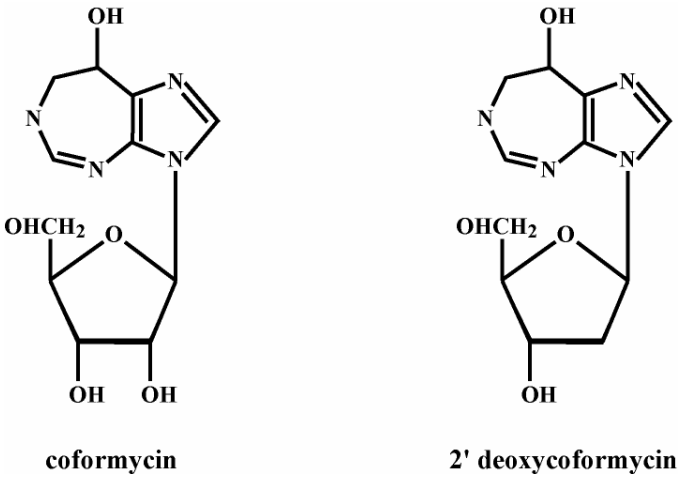

Figure 4 Chemical structure of coformycin and 2' deoxycoformycin.

2' deoxycoformycin is used as pentostatin in cancer treatment.

Toxicity of cordycepin together with deoxycoformycin was studied in beagle dogs. Since the dog is an accepted species for evaluation of drugs intended for use in humans (Haggerty et al., 1992), data from dogs can be used to support the initiation of clinical trials of the drug. Doses of 10 to 20 mg/kg/day of cordycepin given once a day for 3 days in combination with deoxycoformycin were lethal; the maximum tolerated dose was 8 mg/kg/day (Rodman et al., 1997). Lower cordycepin concentrations resulted in cell depletion in the bone marrow, glandular dilation and mucosal necrosis in the stomach, cell depletion and atrophy in the thymus, necrosis in the liver, salivary glands, intestine, and kidney and in the brain. The most severe toxic effects were noted for bone marrow and the gastrointestinal tract. These tissues appear to be the sites of dose-limiting toxicities (Rodman et al., 1997).

1.5.1 Anticancer activity

Cordycepin was the first antitumor antibiotic isolated. Antitumor antimetabolites typically are taken up by cells, metabolised, and subsequently enter cellular nucleotide pools where they exert their effect. Until recently, it was believed that the effectiveness of purine nucleosides and their analogues such as cordycepin in chemotherapeutic regiments was mainly based on their antiproliferative and cytotoxic effect. The antitumor activities of cordycepin in combination with deoxycoformycin were studied in mice infected with B16 mouse melanoma cells. Administration of cordycepin (15 mg/kg body weight) significantly reduced the tumor lump without any loss of bodyweight or systemic actions (Yoshikawa et al., 2004). However, recent studies showed that cordycepin can also exhibit its anticancer activity via programmed cell death (Wu et al., 2007). The realisation that cancer is the result of an imbalance among cell cycle progression and cell death lead cancer research to be increasingly focused on cell cycle and cell death regulatory mechanisms (Foster, 2000; Solary et al., 2000) aiming to specifically induce apoptosis in cancer cells.

1.5.1.1 Radiosensitiser

Potentially lethal damage (PLD) in X-ray irritated mammalian cells in culture can be fixed by a DNA repair mechanism increasing the fraction of surviving cells (Phillips and Tolmach, 1966). A number of studies have been carried out on factors and conditions which modify repair efficiencies especially for their implication in radiation therapy of tumors. Enhanced PLD capacity has been implicated as a major determinant in the radio resistance of malignant human tumors (Boothman et al., 1989). Cordycepin functions as a radiosensitiser in cultured mammalian cells (Robertson et al., 1978) and in particular, purine analogues such as cordycepin are very potent inhibitors of the DNA repair of PLD (Sugahara et al., 1984).

In cultured Chinese hamster V79 cells, cordycepin (50 μM) increased the cytotoxicity after irradiation with γ-rays markedly (Yokoiyama et al., 1992). Cordycepin almost completely suppressed the PLD repair produced by γ-irradiation, possibly by interfering with the repair system (Robertson et al., 1977; Nakatsugawa et al., 1982). Since the repair of PLD resulted in radiation resistance in malignant cells, the inhibition of PLD repair may show promising results in radiation therapy preventing the secondary induction of tumors caused by radiation used in therapy.

1.5.1.2 Activity against TdT^+ cells

Terminal deoxynucleotidyl transferase (TdT) polymerises a single-stranded deoxynucleotidyl sequence without the need of a template (Bollum, 1960). It is expressed in pre-T cells, thymocytes and early pre-B cells (Barton et al., 1976; Hoffbrand et al., 1979) and is considered to play an important role during immunoglobulin and T cell receptor gene rearrangements, thereby increasing the diversity of immunoglobulin and T cell receptor molecules (Desiderato et al., 1984; Yancopoulos et al., 1986). Clinically, over 90% of leukaemic cells in acute lymphocytic leukaemia and approximately 30% of leukaemic cells in the chronic myelogenous leukaemia exhibited elevated TdT activity. The TdT activity of such leukaemic cells is associated with a poor prognosis on chemotherapy and survival time (Benedetto et al., 1986), although the role of TdT in the outcome of leukaemia is not well defined. Newer studies revealed that cordycepin inhibited the activity of TdT very efficiently and thus has been identified as cytotoxic

against TdT^+ cells *in vitro* in the presence of the ADA inhibitor deoxycoformycin, whereas TdT^- cells were significantly less sensitive (Koc and McCaffrey, 1995; Koc et al., 1996). Based on these *in vitro* data, the combination of cordycepin and deoxycoformycin is undergoing Phase I clinical trials in several medical centers in the United States. The mechanism of the distinction of TdT^+ and TdT^- cells has not yet been identified. Cordycepin is phosphorylated and converted to mono-, di-, and triphosphates in both cell types to the same extent (Kodama et al., 2000) and the level of ADA activity in both cells are similarly low, as long as deoxycoformycin was present. This means that the cytotoxicity of cordycepin might be generated through inhibition of TdT. *In vitro* studies showed that cordycepin inhibited the enzymatic activity of human and calf thymus TdT (Tu and Cohen, 1980). The antileukaemic activity of cordycepin combined with deoxycoformycin required TdT positivity of the cells. However, further development of cordycepin as a potential antileukaemic agent for therapy of TdT^+ leukaemia is required.

1.5.1.3 Implications in apoptosis

Apoptosis is a form of programmed cell death and was initially described by its morphological characteristics, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr et al., 1972; Kerr et al., 1994). Mounting evidence indicates that the acquired ability to resist apoptosis is a hallmark of most, and perhaps all types of cancer. It is now clear that some oncogenic mutations disrupt apoptosis, leading to tumor initiation, progression or metastasis and it is well documented that most cytotoxic anticancer agents induce apoptosis, raising the intriguing possibility that defects in apoptotic programs contribute to treatment failure (Lowe and Lin, 2000).

Recently, a relation among polyadenylation and apoptosis has been observed (Thomadaki et al., 2005). PAP II was found to be modulated and polyadenylation to be implicated in the process of apoptosis. PAP II changes during the apoptotic process induced by heat shock or nutrient deprivation, showing dephosphorylation, proteolysis and activity downregulation, which occur relatively early in the process of apoptosis (Atabasides et al., 1998). Along with the induction of apoptosis in different cell lines, the mRNA half-life of key proapoptotic or anti-apoptotic genes is regulated via polyadenylation, with kinetics and intensity differing according to the inducing agent and the cell line examined (Schiavone et al., 2000; Kim et al., 2001). Highly and sustaining

metabolising lymphocytes, as well as less differentiated cells, have higher levels of PAP II activity (Tsiapalis et al., 1998). Overexpression of PAP II mRNA has been demonstrated in chronic leukaemia (Sasaki et al., 1990), as well as in a number of different human tumors originating in breast and pancreas (Bodeker et al., 1998;, Scorilas et al., 1998 and 2000). High PAP II activity levels have been detected in cytosol of breast tumors of untreated patients, associated with the tumor-nodes-metastasis stage of the disease and node invasiveness (Schiavone et al., 2000). It has been suggested that PAP II may be considered as a new, independent, unfavourable prognostic factor for early recurrence and death in breast cancer patients. PAP may represent an aberrantly regulated factor in cancer cells, which supports the main characteristics of their malignant phenotype, such as enhanced proliferative activity and diminished cell turnover via apoptosis (Thomadaki et al., 2005).

Exposure of the cervicial HeLa cells to cordycepin led to a continuous increase in PAP II activity and amount of protein, whereas in the breast cancer cell line MCF-7 cordycepin led to a decrease in both PAP II activity and phosphorylated PAP isoforms (Thomadaki et al., 2005). The MCF-7 cells were arrested in the S phase and HeLa cells in the G2/M phase, which is in agreement with the observed PAP II modifications (Thomadaki et al., 2005). In these two epithelial cancer cell lines PAP II modifications follow cell cycle progression and apoptosis could not be induced by cordycepin.

In studies on the human TdT^+ Molt-4 leukaemia cell line and the Daudi lymphoma cell line, exposure of cordycepin led to growth inhibition and apoptosis induction in both cell types, probably through distinct pathways, because PAP II activity modifications as a response to cordycepin can only be observed in Molt-4 cells, not in Daudi cells (Thomadaki et al., 2007). The increase of PAP II activity and of the amount of isoforms in Molt-4 cells occured prior to any characteristic apoptotic or cytotoxic changes along the early reversible phase of cell commitment to apoptosis (Thomadaki et al., 2007). Different modulations of PAP II in these two cell lines may be a result of the additive effect of the changes in cell cycle and apoptotic pathway induced.

In cells of the Head and Neck Squamous Cell Carcinoma (HNSCC), which is an oral cavity cancer, cordycepin significantly induces cell apoptosis in a time and dose dependent manner (Wu et al., 2007). These cells are arrested in the G2/M phase. Polyadenylation is very important at G1/S phase in order to produce the required mRNAs to further progress in cell cycle. Maybe mitosis can not finish and the cell will arrest at G2/M phase, because not all necessary proteins could be produced during G1/S phase (Wu et al., 2007).

Thus, cordycepin affects PAP II modification tumor cell specifically. The promising observation about the apoptotic effect of cordycepin in HNSCC cells may be a valuable approach in search and development for new anti-oral cancer drugs.

1.5.1.4 Eryptosis induction

Recently it has been unveiled that apoptosis is not only limitated to nucleated cells. Mature enucleated red blood cells (RBC) also share the capacity of self-destruction through apoptosis. Although the mechanism is poorly understood, mature RBCs can undergo a special type of apoptosis known as eryptosis without the involvement of mitochondria and nucleus (Lang et al., 2006). Cordycepin is able to induce eryptosis, as phosphatidylserine externalisation and cleavage of μ-calpain are significantly increased (Lui et al., 2007). An increase in intracellular concentration of [Ca^{2+}] seems to be a crucial factor for the eryptosis induction pathway mediated by cordycepin, as a depletion of the external source of Ca^{2+} results in a reduction of the cordycepin induced eryptosis. At higher cordycepin concentrations (>125 μM), haemolysis is also observed. This erythrotoxic effect of cordycepin implies that it might induce anaemia in cancer patients when it is used in chemotherapy.

1.5.2 Antifungal activity

The ascomycete *Candida albicans* has first been described in1839 and is a normal resident of the gastrointestinal tract of humans and other warm-blooded animals. It is also the most common human fungal pathogen. Although typically asymptomatic, *C. albicans* can proliferate even in healthy people to cause circumscribed infection of the skin, nails and mucous membranes. In patients with deficient immune systems (because of inherited disease, malignancy, concurrent infection, or medical intervention) this same yeast can behave as an aggressive pathogen, attacking virtually any organ system and leading to death in as many as 50% of cases of bloodstream infection (Eggimann et al., 2003). In the past decade, the frequency of diagnosed fungal infections has risen sharply due to several factors, including the increase in the number of immunosuppressed patients resulting from

the AIDS epidemic and treatments during and after organ and bone marrow transplants. Linked with the increase in fungal infections is a recent increase in the frequency with which these infections are resistant to standard antifungal therapy (White et al., 1998). Typical used drugs as treatment against *C. albicans* attack ergosterol biosynthesis or ergosterol containing membranes. Because it is diploid and lacks a complete sexual cycle, conventional genetic analysis is simply not possible. Thus, there is a clear need for a next generation of antifungal agents.

Mice infected with *C. albicans* and treated with cordycepin in combination with deoxycoformycin survived significantly longer and exhibited significantly fewer yeast cells, although kidneys were not completely sterilised (Sugar and McCaffrey, 1998). Interestingly, this effect was unique for cordycepin, as 3' deoxycytidine, 3' deoxyguanidine or 3' deoxyuracil either alone or in combination with deoxycoformycin exhibited no effect on the survival of infected mice. The mechanism of action of cordycepin is not clear. However, identification of useful antifungal drugs with unique modes of action that differ from those of currently available antifungal drugs is desirable since fungi resistant to available agents would likely not be resistant to these newer drugs, and the possibility of combining compounds with differing modes of action may lead to more effective strategies for the treatment of invasive mycoses. Cordycepin may represent a new class of antifungal compounds offering new options for the treatment of fungal infections.

1.5.3 Effect on intestinal bacteria

Various micro-organisms are resident in the human intestinal tract as a highly complex ecosystem and contain a variety of enzymes that perform various types of conversions of bio molecules important for the metabolism in the intestine. Some of these bacteria participate in the normal physiological metabolism such as lactic acid producing bacteria, some contribute to the genesis of diseases by transforming ingested or endogenously formed compounds to harmful agents such as *E. coli*, *Staphylococcus*, *Veillonella*, *Clostridium* and some protect against diseases by generation of beneficial products (Finegold et al., 1975; Modler et al., 1990; Hoover, 1993). Gastrointestinal ecological investigations have demonstrated that there are some differences in intestinal bacteria

between cancer patients and healthy control people, between young and elderly people, as well as between breast and bottle-fed infants (Modler et al., 1990; Moore and Moore, 1995). The microbiota of cancer patients and of elderly people is known to be mainly composed of *Clostridium*. The microbioty in healthy people remains relatively constant, but is known to be greatly influenced by physical, biological, chemical environmental or host factors (Modler et al., 1990; Hoover, 1993).

Nine intestinal bacteria were cultured and exposed to cordycepin. The drug showed strong growth inhibition toward two species of *Clostridium* and *E. coli*, but stimulated growth of four *Bifidobacterium* strains and two *Lactobacillus* strains (Ahn et al., 2000). Daily intake of small amounts of cordycepin might therefore alter the growth and composition of the intestinal flora and modulate the genesis of potentially harmful agents.

1.5.4 Anti-inflammation activity

Interleukin-10 plays a pivotal role in the cytokine network. A large body of evidence supports the view that interleukin-10 shows a strong regulative effect on T-lymphocytes and antigen-presenting cells. The downregulative effect of interleukin-10 on the expression of MHC II antigens, co-stimulatory molecules and adhesion molecules on antigen-presenting cells contributes to the inhibition of antigen presentation, resulting in the abrogation of proliferative responses and cytokine production of responding T cells or T cell clones (Willems et al., 1994; Schandene et al., 1994). Moreover, interleukin-10 inhibits the secretion of proinflammatory cytokines as well and reduces the production of inflammatory mediators like nitric oxide, free radicals and prostaglandin (Moore et al., 1993; Dokka et al., 2001). Hence, Interleukin-10 protects the host against excessive immunopathological and inflammatory processes. Deficiency of interleukin is associated with some diseases such as diabetes mellitus, uveitis and inflammatory bowel disease (Gunnet et al., 2002; Rizzo et al., 1998; Gasche et al., 2000). Although the role of interleukin-10 in health and disease still requires further understanding, preclinical tests have demonstrated that the administration of exogenous interleukin-10 can ameliorate some autoimmune and inflammatory diseases.

Cordycepin was found to strongly increase interleukin-10 production by peripheral blood mononuclear cells in eleven healthy volunteers by enhancing the interleukin-10

mRNA expression (Zhou et al., 2002). In addition, it inhibited interleukin-2 production and T lymphocyte activation. Cordycepin may provide new immunoregulative mechanisms and may be beneficial for the treatment of autoimmune and inflammatory diseases (Zhou et al., 2002).

Activation of macrophages plays an important role in the initiation and propagation of inflammatory responses by the production of cytokines, nitric oxide and other inflammatory mediators. Overexpression of the inflammatory mediators in macrophages is involved in many diseases, such as rheumatoid arthritis (Tilg et al., 1992), atherosclerosis (Coker and Laurent, 1998), chronic hepatitis (Lind, 2003) and pulmonary fibrosis (Bertolini et al., 2001). Thus, the suppression of these mediators may be an effective therapeutic strategy for preventing inflammatory reaction and diseases. Stimulation of macrophages by lipopolysaccharide results in nitric oxide production and the expression of inducible nitric oxide synthetase (iNOS). Cordycepin inhibits nitric oxide production of induced macrophages has a negative impact on iNOS protein and mRNA levels in a dose-dependent manner (Kim et al., 2006). The mechanism of cordycepin appears to involve the inhibition of phosphorylation of NF-κB, Akt and p38, all of which are involved in the production of iNOS and the activation of macrophages. In conclusion, cordycepin might be relevant for clinical use against inflammatory diseases.

1.5.5 Inhibition of human platelet aggregation

Platelet aggregation is absolutely essential for the formation of a haemostatic plug when normal blood vessels are injured. The interaction between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis and myocardial infraction (Schwartz et al., 1990). Inhibition of the platelet-collagen interaction might be a promising approach to the prevention of thrombosis. Thromboxane A_2 formation plays an important role in the mechanism of collagen induced platelet aggregation (Cattaneo et al., 1991), and it also contributes to an increase in cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in collagen activated platelets. An increase in $[Ca^{2+}]_i$ activates both the Ca^{2+}/calmodulin-dependent phosphorylation of myosine light chain and the diacylglycerol-dependent phosphorylation of cytosolic protein to induce platelet aggregation (Kaibuchi et al., 1982). Drugs with an antiplatelet function generally elevate 3',5' cyclic adenosine monophosphate (cAMP),

which results in a decrease of $[Ca^{2+}]_i$ levels (Cho et al., 2007a). Cordycepin has been associated with an inhibitory effect on adenylate cyclase activity, which would suggest a negative function of cordycepin in platelet activation (Londos and Wolff, 1977).

Platelets activated by the thromboxane A_2 analogue U46619 were stimulated with cordycepin leading in the dose-dependent inhibition of platelet aggregation (Cho et al., 2006). $[Ca^{2+}]_i$ significantly decreased (66%) in presence of U46619 and cordycepin, although it is still higher than the basal level. Nevertheless, cordycepin treatment completely blocked the downstream event of Ca^{2+}-dependent phosphorylation giving evidence to the regulatory importance of $[Ca^{2+}]_i$.

In collagen-induced platelets, cordycepin reduced significantly the production of thomboxane A_2, although this effect was not related to inhibition of its metabolic enzymes cyclooxygenase-1 and thromboxane A2 synthetase (Cho et al., 2007b). Inhibition of thromboxane A_2 production was leading to an inhibition of the activation of the downstream enzymes Ca^{2+}/calmodulin-dependent protein kinase and Ca^{2+}-dependent protein kinase C. Ca^{2+} influx through the plasma membrane and release from intracellular storage is normally induced by IP_3, but the production of IP_3 is not altered by cordycepin. Interestingly, cordycepin increased dose-dependently the level of cAMP and cGMP in the presence of collagen (Cho et al., 2007a; Cho, et al. 2007b), which are both strong endogenous negative regulators of platelet aggregation (Park et al., 2004). Cordycepin exerts an inhibitory effect on platelet aggregation which suggests that it may be developed as a novel therapeutic agent for thrombotic diseases such as cardiovascular disease. Other adenosine derivates are also considered as potent antiplatelet agents (Boyer et al., 2002; Xu et al., 2002).

1.5.6 Antiviral activity

Human immunodeficiency virus (HIV) is the causative agent for acquired immunodeficieny syndrome (AIDS) (Barré-Sinoussi et al., 1983). It is a lentivirus that primarily infects $CD4^+$ lymphocytes and cells of the monocyte/macrophage lineage ultimately resulting in diverse immune perturbations and ensuing host susceptibility to severe and life-threatening opportunistic infections (Wong-Staal and Gallo, 1985). As in all retroviruses, an essential feature of HIV replication is reverse transcription of the plus-

strand RNA genome into DNA, a process that requires reverse transcriptase (RT) (Baltimore, 1970). This enzyme is viral encoded and is found associated with genomic RNA in mature HIV virions (Veronese et al., 1985). The relative restriction of RT to retroviruses and viruses requiring a short reverse transcription step makes RT an important target for antiviral and particular antiretroviral therapeutic intervention. The step of reverse transcription is blocked by the 2',5' oligoadenylate (2-5A) system, which is part of the host's natural antiviral response induced by interferons (Lengyel, 1982). Interferons increase 2',5' oligoadenylate synthetase gene expression resulting in increased synthesis of 2-5A. The produced oligoadenylate activates a latent endoribonuclease (Rnase L) that cleaves viral and cellular RNA and thereby inhibits protein synthesis.

The short half-life of authentic 2-5A in biological systems is a disadvantage in the control of viral replication (Doetsch et al., 1981). Modification of 2-5A at the 3' hydroxyl groups provides various 2-5 A analogues with remarkably increased metabolic stability to cellular nucleases, while maintaining the ability to activate Rnase L (Nyilas et al., 1986). Consistent with its metabolic stability and biological activity the cordycepin 2-5 A analogues inhibit the reverse transcriptase more efficiently than the authentic 2-5 A (Montefiori et al., 1989). Cordycepin *per se* does not change the activity of reverse transcriptase nor does it show an antiviral activity in HIV-infected MT-2 cells, indicating that the cordycepin analogues of 2-5 A did not serve as a prodrug of cordycepin (Montefiori et al., 1989). From all tested analogues, Co-Co-Co and pCo-Co-Co are the most efficient inhibitors of reverse transcription, while other cellular DNA polymerases (α, β and γ) are insensitive (Müller et al., 1991).

1.5.7 Activity against *Leishmania* and *Trypanosoma* species

Leishmania and *Trypanosoma* species are parasitic protozoa of the order Kinetoplastida that cause Leishmaniasis and African sleeping sickness (Bloom, 1979). Both diseases are prevalent in various tropical regions of the globe and by infecting millions of people prominent causes of illness and death in endemic areas. Leishmaniasis or African sleeping sickness is initiated when sand flies or tsetse flies, respectively, inject the extracellular promastigote form of the parasite into the skin. The promastigotes are phagocytised by macrophages and transform into the amastigote stage, which is responsible for clinical

disease. Treatment and control of these important tropical diseases are compromised by the toxicity and expense of current drugs, the slow development of novel therapies and the increasing emerge of drug-resistant strains (Geary et al., 1986).

The transport of nucleosides and nucleobases across the plasma membrane of parasitic protozoa has elicited considerable attention due to the central role of purine metabolism in these microorganisms. Unlike their vertebrate hosts, all parasitic protozoa examined are unable to synthesise purines *de novo* and, hence, rely completely on the salvage of these compounds from their hosts (Berens et al., 1995; Ullmann, 1984). The first step in these salvage pathways is the transport of the preformed purines across the surface membrane of the parasite, underscoring the importance of nucleoside and nucleobase permeases for parasite nutrition. Treatment, of course, tries to profit from this vulnerability. Drugs like Allopurinol are taken up by the parasite, metabolised by salvage enzymes allowing the end product to enter the nucleoside triphosphate pool of the parasite, whereas the corresponding interconversions do not occur efficiently in mammalian cells. *Leishmania donovani* possesses two distinct nucleoside transporters, which have K_m values in the submicromolar range for adenosine and inosine, revealing that the parasite uptake systems have 100- to 1000-fold lower K_m values than typical mammalian nucleoside transporters (Aronow et al., 1987). Related studies identified two distinct transport activities in African trypanosomes, which are driven by protonmotive force being able to concentrate adenosine inside the cells (Carter and Fairlamb, 1993; de Koning et al., 1998). On the contrary, mammalian cells have both facilitated diffusion carriers and Na^+/adenosine co-transporters with much lower affinities (Thorn and Jarvis, 1996). These results underscore the efficiency with which the parasite transporters can compete with the host for essential purines and also the efficacy of agents for delivering cytotoxic purine analogues to the parasite. The current believe is, that adenosine is salvaged by a two-step process in *Trypanosoma brucei* where intracellular cleavage to adenine is followed by phosphoribosylation to AMP (El Kouni, 2003).

The prodrug cordycepin would be efficiently conveyed by the parasite, converted into CoTP and could then inhibit growth of the parasite. *In vitro* assays with purified RNA polymerase III from *Leishmania mexicana* exhibit an inhibition of the enzymatic activity by CoTP (Nolan and Fehr, 1987).

In a mouse model, administration of 2 mg/kg body weight of cordycepin in combination with ADA inhibitor deoxycoformycin completely eliminated parasites levels of *T. brucei* in the blood (Rottenberg et al., 2005). This auspicious treatment can also affect

the outcome of infection when administered after the parasite has penetrated into the brain parenchyma and can completely purge the mice from parasites.This data strongly supports further development and testing of nucleoside analogues as therapeutic agents for human African trypanosomiasis and Leishmania.

2 Transcription by RNA polymerase II

The genome of eukaryotes is transcribed by three different highly conserved RNA polymerases. The ribosomal RNA (rRNA) is transcribed by RNA polymerase I, whereas RNA polymerase III transcribes several types of RNAs: the 5S rRNA, transfer RNAs (tRNAs) and other small RNAs. RNA polymerase II is the most versatile enzyme of the three and synthesises pre-messenger RNA (pre-mRNA) of protein-coding genes and most of the small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and other non-coding RNAs.

2.1 RNA polymerase II transcription cycle

In eukaryotes, the completion of messenger RNA (mRNA) synthesis is not simply the copying of the genetic information from the DNA template into an RNA transcript. Before the mRNA is ready to be transported out of the nucleus, it has to undergo three major processing steps to produce a mature and translatable mRNA. These three steps comprise the acquisition of the cap structure at the 5' terminus, splicing out the introns within the body of the pre-mRNA and the generation of the 3' end, which includes cleavage and the addition of a poly(A) tail. Therefore, the generation of mature mRNA by RNA Polymerase II (RNA Pol II) involves multiple processes, some occur sequentially and others in parallel. Transcription by RNA Pol II is a cyclic process composed of five primary phases: promoter binding and pre-initiation, initiation, promoter clearance, elongation and RNA transcript termination and release. Regulation of transcription can occur at each of these steps. Recent studies in yeast point to the existence of gene loops, in which a termination region of a transcriptionally active gene can be physically linked to its promoter site (O'Sullivan et al., 2004; Ansari and Hampsey, 2005). In such a case, termination can serve to facilitate transcriptional reinitiation.

Transcription starts in the yeast *S. cerevsisiae* with the assembly of the pre-initiation complex (PIC) at the promoter site of the DNA. The PIC consists besides RNA Pol II of the general transcription factors TFIID, TFIIB, TFIIE, TFIIF, TFIIH, the mediator complex and additional cofactors (Orphanides et al., 1996). The ATP-dependent process of melting of the double-stranded DNA into a singe-stranded bubble requires the action of the

general transcription factors TFIIE and TFIIH (Goodrich and Tjian, 1994; Holstege et al., 1996). Generation of the open complex of DNA and PIC is a prerequisite for the process of transcription.

Transcription initiation occurs upon addition of the two initiating nucleoside triphosphates (NTPs) complementary to the DNA sequence and the formation of the first phosphodiester bond.

Before RNA Pol II enters the process of productive transcription elongation, it has to pass a stage known as promoter clearance. During this process, the PIC is partially disassembled. All general transcription factors except TFIIF remain at the promoter serving as a scaffold for a next round of PIC formation (Yudkovsky et al., 2000). Reinitiation of transcription therefore is a faster process relative to the initial round (Orphanides and Reinberg, 2000).

The earliest stages of transcription are marked by instability of the transcription complex and a tendency to release the RNA. Appearance of stalled RNA Pol II-DNA complexes and short RNA products during promoter clearance indicates that immediately after transcription starts, a stable transcription elongation complex (TEC) has not formed. Biochemical studies have determined that RNA Pol II-TECs are unstable before the RNA-DNA hybrid reaches 8 nt in length (Kireeva et al., 2000). Crystal structure of the yeast RNA Pol II-TEC containing a 14 nt RNA shows that the RNA-DNA hybrid within the transcription bubble is 9 nt long (Gnatt et al., 2001).

During early elongation, the capping of the 5' end of the nascent pre-mRNA is carried out. The first nucleotide of the RNA is converted to a diphosphate by the 5' triphosphatase Cet1p (Tsukamoto et al., 1997) followed by the fusion with a GMP moiety catalysed by the guanylyltransferase Ceg1p (Itoh et al., 1984). Finally, the methyltransferase Abd1p methylates the GMP at N7 position completing the cap structure (Mao et al., 1995).

During the process of transcription elongation, emerging introns are removed immediately. This event is called splicing and is a crucial and ubiquitous step in eukaryotic gene expression. It is carried out by the humongous splicosome complex which is composed of more than 200 different proteins and the RNA components U1, U2, U4, U5 and U6, which are called small nuclear RNAs, or snRNAs (Nilsen, 2003). The splicosome forms on the intact precursor RNA after the 5' splice site and branch site has been recognized by U1 and U2 snRNA, respectively. Within the assembled splicosome, intron excision occurs in two chemical steps: first the 5' splice site cleavage and lariat formation

is carried out, then the 3' splice site is cleaved and the exons are ligated. After the exon ligation, spliceosome disassembly frees its components for the *de novo* synthesis of further spliceosomes (Jurica and Moore, 2003). The excised RNA lanat is rapidly degraded.

The final stage in the transcription process allows on one hand the release of the transcript from the site of transcription and on the other hand the release of RNA Pol II from the DNA template. This affords recycling of the RNA Pol II enzyme for a further round of transcription. Cleavage and polyadenylation of the mRNA precursor is essential for the transport of the mature mRNA out of the nucleus and its translation and stability (Ford et al., 1997; Wickens and Stephenson, 1984).

2.2 CTD phosphoryation cycle

With the step of promoter clearance, the beginning of cycling events within the transcription cycle starts: the phosphorylation of the highly conserved C-terminal domain (CTD) of Rpb1, the largest subunit of RNA Pol II (Allison et al., 1985) (figure 5). The tail-like CTD protrudes from the catalytic core of RNA Pol II and is flexibly located at the exit groove of the pre-mRNA. The CTD is composed of heptapeptide repeats with the consensus sequence YSPTSPS. The number of the repeats varies species-dependently,

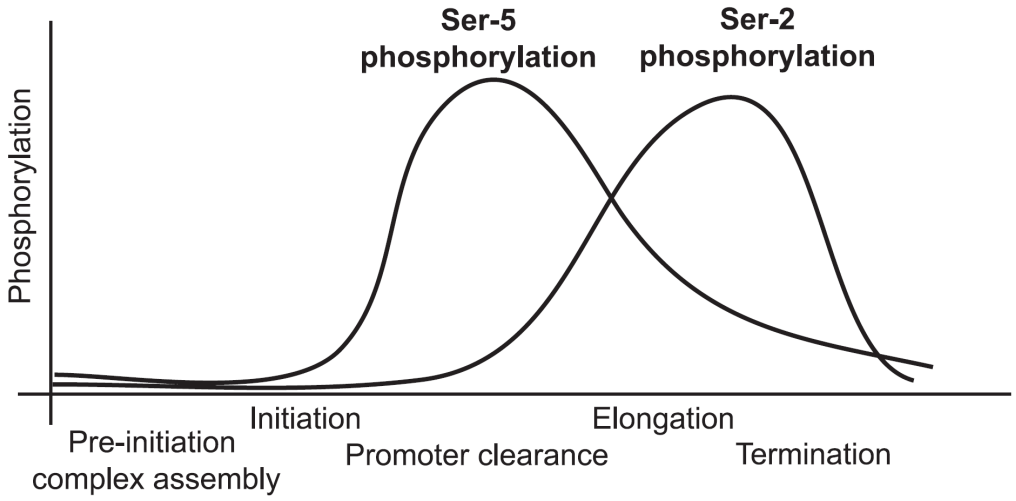

Figure 5 Phosphorylation of the CTD during the transcription cycle (Svejstrup, 2004).

The graph shows the phosphorylation levels at Ser 2 and Ser 5 of the CTD during the transcription cycle.

consists of 27 repeats in the yeast *S. cerevisiae* (Allison et al., 1985) and 52 in mammals (Cordon, 1990). These repeats can be heavily modified by reversible phosphorylation at serine 2 (Ser 2) and serine 5 (Ser 5) (Christmann and Dahmus, 1981), allowing four possible phosphorylation states: unphosphorylated, phosphorylated at Ser 2, phosphorylated at Ser 5, and phosphorylated at both sites, Ser 2 and Ser 5.

In contrast to its simple repeated sequence, the function of the CTD is very complex and it is involved in all steps of mRNA formation (Dahmus, 1995). RNA Pol II phosphorylated at Ser 5 associates with the transcription step of initation and early elongation and it is reduced towards the 3' region. In contrast, the amount of phosphorylated Ser 2 increases towards the 3' end during elongation and marks a polymerase at late elongation or termination (Komarnitsky et al., 2000; Cho et al., 2001). At the preinitiation step, the CTD remains unphosphorylated (figure 5).

In *S. cerevisiae*, the Kin28p subunit of the general transcription factor TFIIH bound to the cyclin CDK7 phosphorylates the CTD at Ser 5 (Feaver et al., 1994; Rodriguez et al., 2000), whereas the cyclin-dependent kinases Bur1p/Bur2p and Ctk1p/Cdk9p hold a kinase activity for Ser 2 phosphorylation (Keogh et al., 2003; Cho et al., 2001) . Fcp1p is thought to play a major role in the recycling of RNA Pol II by dephosphorylating Ser 2 (Hausmann and Shuman, 2002). The second identified CTD-phosphatase is the Ser 5 phosphatase Ssu72p, which is also part of the cleavage and polyadenylation factor (Krishnamurthy et al., 2004). The action of CTD kinases and phosphatases are necessary for RNA Pol II to trigger the next step of the transcription cycle. Therefore, this balanced action of CTD kinases and phosphatases is highly regulated. With such an important role in transcription, it is not surprising that the CTD is essential for viability (Allison et al., 1988). The CTD is a key element in guiding the polymerase through the transcription cycle and in coupling the transcription process to mRNA processing events. Thus, the CTD plays an active role in all transcription processes, both by recruiting the RNA-processing factors to the nascent transcript and by directly activating them (Prelich, 2002). The detection of CTD-binding domains for several capping (Cho et al., 1997), splicing (Yuryev et al., 1996), and 3' end processing factors (Barilla et al., 2001) suggests that the CTD serves as an assembly platform for processing factors. Moreover, most of the mRNA processing steps occur cotranscriptionally and many events during the synthesis of a mature mRNA are co-regulated (figure 6) (Maniatis and Reed, 2002; Orphanides and Reinberg, 2002).

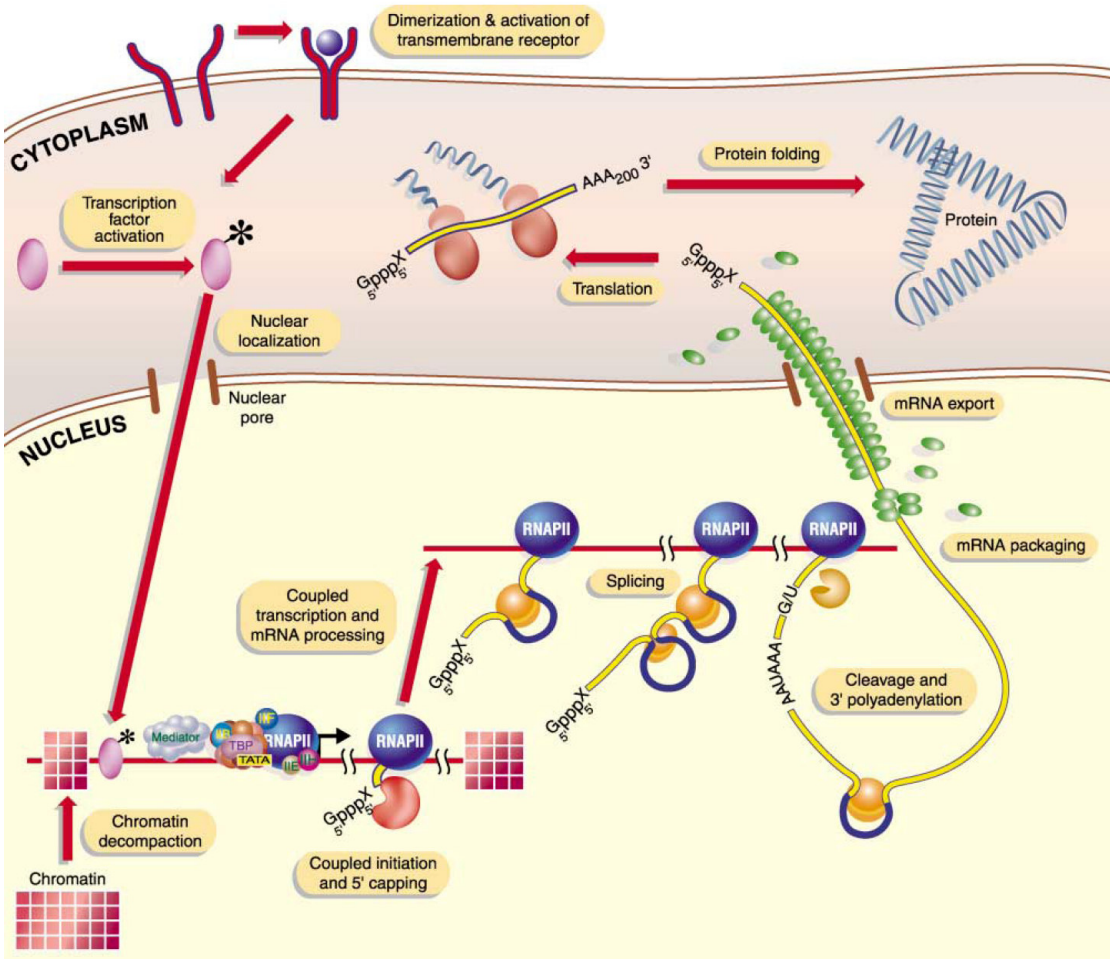

Figure 6 Linking pre-mRNA processing to the RNA Pol II transcription cycle (Orphanides and Reinberg, 2002).

In the traditional view of gene expression, each step has been referred to an individual event, and the completion of it triggers the next step in the row. In contrast to that, the contemporary view shows gene expression as a subdivision of continuous processes, which are physically and functionally connected. The CTD plays a pivotal role in coordinating the transcription and processing events.

2.3 Chromatin modification and transcription

In eukaryotic cells, DNA is assembled into chromatin, a highly compacted nucleoprotein complex. The basic unit of chromatin, the nucleosome, is an octamer of core histones (two of each H2A, H2B, H3 and H4) wrapped around 147 bp of DNA. Chromatin provides DNA compaction and thereby adds another level of complexity to the regulation of the

processes requiring protein access to DNA. Chromatin remodeling is dynamic, and chromatin structure is altering so that transcription machinery has access to previously condensed DNA. Chromatin remodeling complexes use ATP to mechanically rearrange nucleosomes. There are 7 major chromatin-remodeling complexes in yeast: SWI/SNF, RSC, INO80, CHD1, Isw1a, Isw1b, and Isw2, each are containing a related ATPase catalytic subunit (Uffenbeck and Krebs, 2006). The mechanisms by which these complexes rearrange nucleosomes are various, enabling both general transcriptional regulation and promoter-specific regulation. It has also been demonstrated that chromatin remodelers are able to regulate transcription at the level of elongation (Morillon et al. 2003), and perform regulatory functions via novel remodeling activities, such as histone exchange and (or) histone variant deposition (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004).

Histones are posttanslationally and covalently modified at the protruding and therefore highly accessible N-terminal tails by a wide variety of enzymes. These modifications include acetylation, methylation, ubiquitination and phosphorylation. Histone modification is another mechanism used by cells to control access to DNA in chromatin. Depending on the type of modification, changes in electronegativity can occur, which can alter the DNA–histone binding affinity and stabilising or destabilisng the chromatin structure. Histone modification also generates binding surfaces for other factors, such as chromatin remodelers, repressors, or transcription factors (Spencer and Davie, 1999). The large number of possible histone modification allows highly specific and complex signaling mechanisms and some particular histone modification can be associated to general transcriptonal effects. For example, the acetylation via histone acetyltransferases (HATs) is associated with active transcription (Struhl, 1998), while deacetylation through histone deacetylase (HDAC) complexes normally results in gene repression (Kadosh and Struhl, 1998). Lysine acetylation affects chromatin by imparting a negative charge and neutralizing the interaction between the histone tail and the phosphate backbone of DNA and creates a novel binding site that is bound by factors containing bromodomains. Histone acetylation is typically enriched at transcriptionally active genes, because it destabilises the chromatin structure (Svejstrup, 2007).

2.4 Transcription termination

Transcription termination is critical for the overall maintenance and integrity of the transcriptome and it plays an important role in the regulation of gene expression. RNA Pol II is able to terminate polyadenylation-dependently as for the mRNA and as well polyadenylation-independently for snRNA, snoRNA and cryptic unstable transcripts (CUTs).

2.4.1 Polyadenylation-dependent termination

Termination is functionally connected with cleavage and polyadenylation of the nascent transcript (Zaret and Sherman, 1982). In the two-step pre-mRNA 3' processing reaction, transcription of the poly(A) signal triggers the endonucleolytic cleavage of the nascent transcript generating an upstream cleavage product, which is immediately polyadenylated

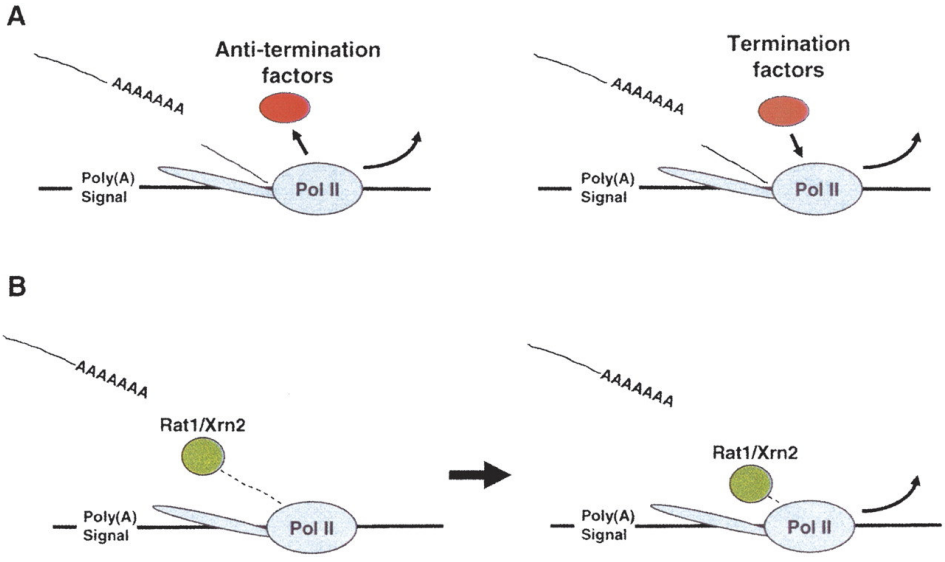

Figure 7 Models for transcription termination by RNA Pol II (Rosonina et al., 2006).

A) *Antiterminator or allosteric model. Termination is achieved either by the release of anti-termination factors or by binding termination factors triggering a conformational change of RNA Pol II to a less processive state.* ***B)*** *Endonucleolytic cleavage of the nascent RNA transcript provides a free 5' end which serves as an entry point of the exonuclease Rat1p. When Rap1p reaches the RNA Pol II complex, it induces termination and RNA Pol II dissociates from the DNA template.*

(Colgan and Manley, 1997). The remaining downstream cleavage product having an uncapped 5' end is highly unstable and is rapidly degraded (Manley et al., 1982).

Two models exist addressing the mechanistic basis for the connection between termination on one hand and cleavage and polyadenylation on the other: the "torpedo" and the "allosteric" or "antiterminator" model (figure 7) (Rosonina et al., 2006). In the latter model, transcription termination is caused by the destabilisation and/or a conformational change of the RNA Pol II elongation complex after transcribing the poly(A) site. Release of antitermination factors or recruitment of termination factors triggers dissociation from the DNA template (Logan et al., 1987). The torpedo model postulates that endonucleolytic cleavage at the poly(A) site creates an entry site for the 5' → 3' exonuclease Rat1p, which degrades the RNA downstream of the cleavage site. The exonuclease continues degrading the transcript until it reaches the RNA Pol II elongation complex and destabilises it by inducing an arrest and promotes termination (Connelly and Manley, 1988). Although both models rely on recognition of the poly(A) site, the fundamental difference is that only the torpedo model depends on the successful endonucleolytic cleavage of the nascent RNA to create an entry site for Rat1p.

A hybrid model for coupling termination with 3' end processing has been proposed, where aspects of both models are combined, since neither the exonuclease Rat1 nor the binding of cleavage and polyadenylation factors alone were sufficient to cause a poly(A) site dependent termination (Luo et al., 2006). According to the hybrid model, the association of Rat1p and CPF on the CTD of the RNA Pol II elongation complex results in an allosteric change in the polymerase which favours termination and endonucleolytic cleavage is stimulated by associated 3' end processing factors.

2.4.2 Elements of polyadenylation-dependent termination

Termination of RNA Pol II transcription is initiated by the cotranscriptional recognition of *cis*-acting sequences in the 3' end of the nascent transcript by a multi-component complex carrying out cleavage and polyadenylaton.

2.4.2.1 *Cis*-acting elements

The *cis*-acting elements, sequences on the RNA precursor, determine the site of cleavage and polyadenylation. The signals for 3' end formation in yeast are less well conserved than the poly(A) site signals in higher eukaryotes (Wahle and Kuhn, 1997). However, there are common elements in the untranslated region (UTR) of genes, which guide the site of cleavage and polyadenylation (see figure 8 on page 37)

The efficiency element is found at variable distances upstream of the cleavage site and often contains alternating AU dinucleotides or U-rich stretches. Computer analysis has shown that more than half of approximately 1000 examined nuclear genes in yeast contain the sequence UAUAUA in the 3' UTR (Graber et al., 1999). The U residues at the first and the fifth positions have been identified as the most critical nucleotides in this sequence (Guo and Sherman, 1995).

The positioning element is located approximately 20 nucleotides upstream of the cleavage and polyadenylation site. It consists of an A-rich stretch and sequences as AAUAAA or AAAAA have been identified as most efficient (Guo and Sherman, 1995). The positioning element also contributes to 3' end processing and poly(A) site selection (Abe et al., 1990; Hyman et al., 1991; Irniger et al., 1992). The U-rich elements are found immediately upstream or downstream of the poly(A) site (Graber et al., 1999). They contribute to poly(A) site recognition and enhance processivity of 3' end formation (Dichtl and Keller, 2001). The poly(A) site preferentially is a Pyrimidine(A)n, and might itself act as a polyadenylation signal (Heidmann et al., 1992).

2.4.2.2 Factors involved in 3' end processing

Proteins that are sufficient to perform 3' end formation *in vitro* have been identified (Chen and Moore, 1992). For the cleavage reaction, the cleavage and polyadenylation factor IA (CF IA), cleavage and polyadenylation factor IB (CF IB), and the cleavage factor II (CF II) are sufficient, while specific poly(A) addition requires in addition the polyadenylation factor (PF I) and the poly(A) binding protein (Pab1). It was shown that CF II and PF I form a functional unit *in vivo* (Ohnacker et al., 2000) and therefore this factor has been renamed as cleavage and polyadenylation factor (CPF) (figure 8).

Cleavage and polyadenylation factor IA (CF IA)

CF I was originally detected as an activity needed for cleavage and polyadenylation reaction *in vitro* (Chen and Moore, 1992) and further purification identified two components, CF IA and CF IB (Kessler et al., 1996). CF IA consists of four proteins: Rna14p, Rna15p, Pcf11p and Clp1p. Yeast strains harbouring temperature-sensitive mutations in the essential genes *RNA14* or *RNA15* show a dramatic poly(A) tail shortening (Minvielle-Sebastia et al., 1991). These mutations are synthetically lethal with mutations in the *PAP1* (poly(A) polymerase) gene (Minvielle-Sebastia et al., 1994). Extracts from *RNA14*-or *RNA15*-mutant strains are defective in cleavage and polyadenylation (Minvielle-Sebastia et al., 1994) indicating that they are involved in both steps of 3' end processing. The same malfunction has also been reported for extracts of strains carrying temperature-sensitive mutations in *PCF11* gene (Amrani et al., 1997). Pcf11p contains a CTD interaction domain binding the Ser 2 phosphorylated CTD and couples 3′ end processing to transcription (Barilla et al., 2001; Noble et al., 2005). The Clp1p component interacts with Pcf11p (Gross and Moore, 2001) and Ysh1p of CF II (Kyburz et al., 2003). Crystal structure of Clp1-ATP with the Clp1-binding site of Pcf11p was unable to explain the function of ATP binding and/or its hydrolysis by Clp1p in the process of 3' end formation (Noble et al., 2007).

Cleavage and polyadenylation factor IB (CF IB)

CF IB is a single polypeptide encoded by the *NAB4*/*HRP1* gene (Kessler et al., 1997). Nab4p is an essential heterogeneous nuclear ribonucleoparticle (hnRNP) that can shuttle in and out of the nucleus (Kessler et al., 1997). It is unclear, if Nab4p is involved in the cleavage reaction itself or if it is only required for regulating cleavage site utilisation (Minvielle-Sebastia et al., 1998). *In vitro* cleavage reactions lacking *NAB4* produced an increase in the usage of cryptic cleavage sites leading to the hypothesis that Nab4p is involved in the discrimination between correct and cryptic cleavage sites (Minvielle-Sebastia et al., 1998). In addition to its role in 3′ pre-mRNA processing, Nab4p has been implicated in mRNA export and nonsense-mediated decay (Gonzalez et al., 2000).

Cleavage and polyadenylation factor (CFP)

CPF contains all subunits of CF II and PF I and in addition the proteins Ref2p, Pti1p, Swd1p, Glc7p, Ssu72p and Syc1p. These six proteins are combined in a further subcomplex of CFP named associated with Pta1 (APT), because it was observed by

tagging different subunits of CPF that not all proteins pulled down the entire CFP complex (Nedea et al., 2003). Ref2p is a non-essential RNA binding protein that is important for the efficient cleavage of pre-mRNAs with weak poly(A) sites (Russnak et al., 1995). Furthermore, it has been implicated in poly(A)length control and snoRNA 3' end maturation (Dheur et al., 2003; Magnus et al., 2004).

Glc7p is the catalytic subunit of type 1 protein phosphatase (Feng et al., 1991) and is involved in the regulation of many different physiological processes, e.g. sporulation, glycogen metabolism and mitosis. It plays an essential role in snoRNA transcriptional termination and for that the roles of two other APT components, Swd2p and Ref2p, are to keep Glc7p associated with the complex (Nedea et al., 2008). Depletion of Glc7p causes shortened poly(A) tails *in vivo* and *in vitro* and Glc7p is not essential for cleavage at the poly(A) site, but for poly(A) addition (He and Moore, 2005).

The CTD phosphatase Ssu72p (Krishnamurthy et al., 2004) is an essential protein and was initially identified based on genetic interaction with the general transcription factor TFIIB (Sun and Hampsey, 1996). It interacts physically with TFIIB (Wu et al., 1999), the Rpb2 subunit of RNA Pol II (Dichtl et al., 2002a), TFIID (Sanders et al., 2002) and TFIIH (Ganem et al., 2003). This would suggest a role for Ssu72p in transcription initiation, however as component of CFP, defective forms of Ssu72p affect cleavage (He et al., 2003) and termination (Steinmetz and Brow, 2003) but it is dispensable for polyadenylation.

Swd2p is an essential protein. It is a subunit of CPF as well as of the COMPASS-complex, which methylates K4-H3 (Roguev et al., 2001). Swd2p is required for global histone methylation on K4-H3, for RNA Pol II termination on specific genes, but not for 3' end processing (Dichtl et al., 2004; Cheng et al., 2004).

Cleavage factor II (CF II)

CF II consists of four essential proteins: Cft1p/Yhh1p, Cft2p/Ydh1p, Brr5p/Ysh1p and Pta1p. Yhh1p and Ydh1p are both RNA binding proteins that are involved in poly(A) site recognition (Dichtl et al., 2002b; Kyburz et al., 2003). Both proteins interact with phosphorylated CTD of RNA Pol II, and they may therefore mediate the binding of CFP to the CTD, which is important for the coupling of transcription and 3' end formation (Bentley, 2002). *YHH1* mutant strains show a defect in RNA Pol II termination. Ysh1p belongs to a large family of metallo-hydrolytic enzymes that have the ability to cleave nucleic acids (Callebaut et al., 2002) and it is thought that Ysh1p might be the

endonuclease that cleaves the pre-mRNA at the poly(A) site (Chanfreau et al., 1996; Jenny et al., 1996). Pta1p is reported to be involved in poly(A) addition but not in cleavage (Preker et al., 1997), but another study found its implication in both steps (Zhao et al., 1999). Glc7p depleted extracts revealed a defect in poly(A) addition and in the accumulation of phosphorylated Pta1p, leading to the suggestion that the activity of Pap1p might be regulated, directly or indirectly, by phosphorylation of Pta1p (He and Moore, 2005).

Polyadenylation factor I (PF I)

PF I was initially identified as an activity, which supported poly(A) addition but not cleavage. It contains the proteins Yth1p, Fip1p, Pap1p, Mpe1p and Pfs2p, which are all essential. Yth1p is implicated in both, cleavage and polyadenylation (Barabino et al., 2000). The protein bears five zink fingers having the ability to bind RNA (Barabino et al., 1997). Moreover Yth1p might play an important role for transition from cleavage to polyadenylation (Tacahashi et al., 2003). Fip1p interacts with Pap1p (Preker et al., 1995). The interaction between Fip1p and Rna14p (Preker et al., 1995) is thought to link the two large subcomplexes within the 3′ end processing machinery: CFI, which binds the upstream A-rich motif in the pre-mRNA (Gross and Moore, 2001), and CPF, which contains the poly(A) polymerase and Ysh1p, the nuclease responsible for pre-mRNA cleavage. The protein Fip1p is largely disordered in the absence of Pap1p, and this suggest that only a portion of Fip1p folds upon binding the polymerase (Meinke et al., 2008). Phosphorylated CF II might weaken the binding of Fip1p to Pap1p, which could have an influence on polyadenylation efficiency (He and Moore, 2005).

Pap1p catalyzes the addition of a polyadenosine tail to pre-mRNAs and was the first purified factor of the 3’ end machinery. The yeast and mammalian Pap1p proteins are 47% identical within the first 400 amino acids thought to comprise the nucleotidyl transferase activity (Martin and Keller, 1996). Yeast Pap1p is not required for cleavage *in vitro*, however, temperature-sensitive mutants show a change in poly(A) site usage in *ACT1* transcript (Mandart and Parker, 1995). The crystal structure of Pap1p has been solved alone and in complex with cordycepin (Martin et al., 2000; Bard et al., 2000).

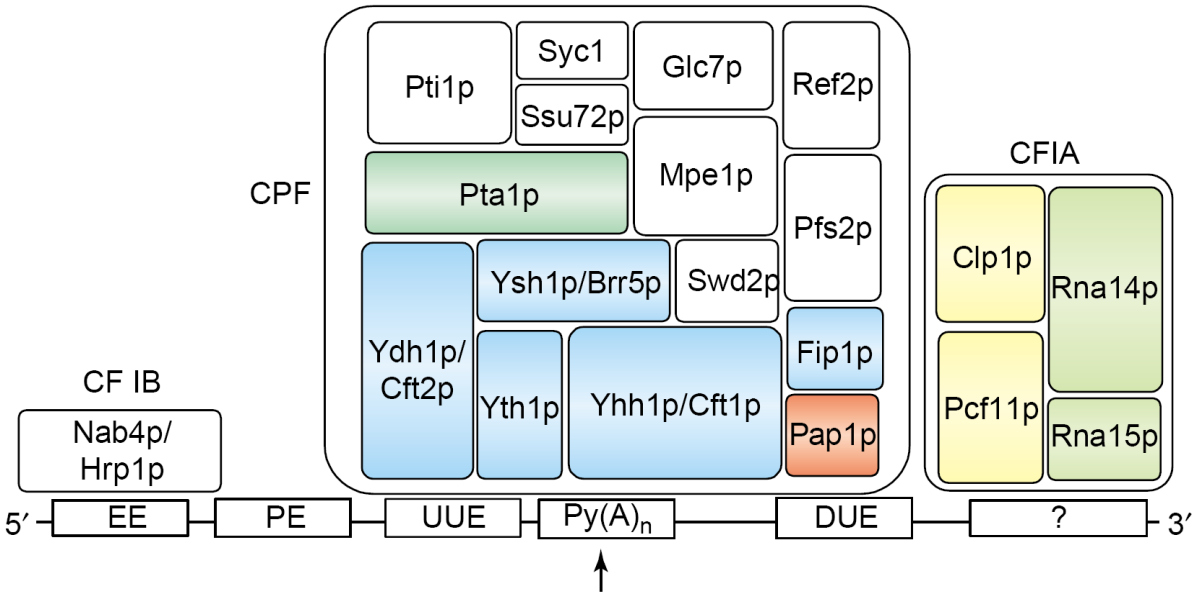

Figure 8 Diagram showing the *cis*- and *trans*-acting elements required for transcription termination and 3' end processing in yeast (Proudfoot, 2004)

Abbreviations used in this scheme: EE efficiency element; PE positioning element; UUE upstream U-rich; DUE downstream U-rich element. The array displays the poly(A) site.

2.4.3 Polyadenylation independent termination

Transcription termination of snRNA and snoRNA depends on a protein complex called Nrd1p-complex containing the RNA binding proteins Nrd1p and Nab3p and some additional co-factors (Steinmetz et al., 2001). Like components of the polyadenylation-dependent termination, the Nrd1p-compex interacts directly with RNA Pol II through a CTD-interaction domain on Nrd1p. Two common RNA sequence motifs are recognised by the Nrd1p-complex and play a significant role in defining polyadenylation-independent termination: the sequence GUA[AG] corresponds to the Nrd1p-binding site and Nab3p binds specifically to the UCCU motif (Carroll et al., 2004). Efficient termination of snRNA and snoRNA transcription requires the c*is*-element which is recognised by Nrd1p and Nab3p, the putative RNA helicase Sen1p and an intact CTD of RNA Pol II (Steinmetz et al., 2001). The primary snRNA and snoRNA transcripts contain extended 3' ends that are trimmed by the nuclear RNA exosome. This complex with 3'→5' exonucleolytic activity is able to degrade unstructured stretches of RNA and trimming proceeds until the complex encounters specific protein bound RNA elements at the 3' end of the mature snRNA and snoRNA (Allmang et al., 1999; van Hoof et al., 2000). The activity of the exosome is stimulated by the TRAMP complex (LaCava et al., 2005), consisting of a recently

identified poly(A) polymerase Trf4p (or Trf5p) (Vanacova et al., 2005; Wyers et al., 2005), Mtr4p, a putative RNA helicase previously implicated in the activation of the nuclear exosome (de la Cruz et al., 1998) and Air1p/Air2p. TRAMP-mediated polyadenylation of RNA destabilised the RNA by marking it for degradation by the nuclear exosome (Egecioglu et al., 2006). The Nrd1p-complex copurifies with both the nuclear exosome and TRAMP (Vasiljeva and Buratowski, 2006). Interestingly, Nrd1p has been shown to bind strongly Ser 5 phosphorylated CTD *in vitro* and chromatin immunoprecipitation experiments revealed that Nrd1p is recruited to RNA Pol II at the 5' end of the gene (Vasiljeva, et al., 2008).

The importance of coupling transcription termination and proper 3' end formation has been illustrated by the discovery of the from intergenic DNA regions abundantly transcribed but scarcely detectable cryptic unstable transcripts (CUTs) (Wyers et al., 2005). These transcripts are hardly detectable in wild-type strains, but can be visualised by disrupting either *TRF4* the gene encoding the poly(A) polymerase subunit of the TRAMP complex or *RRP6*, a nonessential subunit of the nuclear exosome (Mitchell and Tollervey, 2000). RNA Pol II termination of CUT transcripts also involves the Nrd1p-complex. However, CUTs are rapidly degraded by polyadenylation by the TRAMP complex and subsequent action of the exosome. Because of the lack of any protective element, they are completely degraded by the nuclear exosome. This mechanism identifies the implication of the TRAMP complex together with the nuclear exosome in transcription quality control. CUT transcription has been shown to interfere with promoters of coding regions and hence regulates gene expression. The expression of *IMD2*, *SER3* and *PHO5* are regulated by transcription of a CUT. *IMD2* encodes an enzyme important for GTP biosynthesis and *IMD2* mRNA levels correlate inversely with GTP levels (Exinger and Lacroute, 1992). When GTP levels are high, transcription starts from G-start sites (TATA box proximal) resulting in the synthesis of noncoding transcripts that prevent initiation for *IMD2* production. Low GTP levels allows the usage of the A start site (TATA box distal) and the production of *IMD2* mRNA (Kuehner and Brow, 2008). *SER3* encodes a serine biosynthetic enzyme and its expression is regulated by the CUT *SRG1*, which covers the promoter of *SER3* (Martens et al., 2004). *SRG1* transcription is induced by high serine levels resulting in repression of *SER3*; in lack of serine, *SRG1* transcription is dramatically reduced allowing *SER3* expression (Martens et al., 2005). In the case of *PHO5*, the noncoding antisense transcript is transcribed at low levels and functions in *PHO5* activation and not repression (Uhler et al., 2007).

RNA Pol II has the choice between different transcription termination modes and the polymerase has to be directed to proper termination by cis-acting elements and the pooling of necessary factors (figure 9).

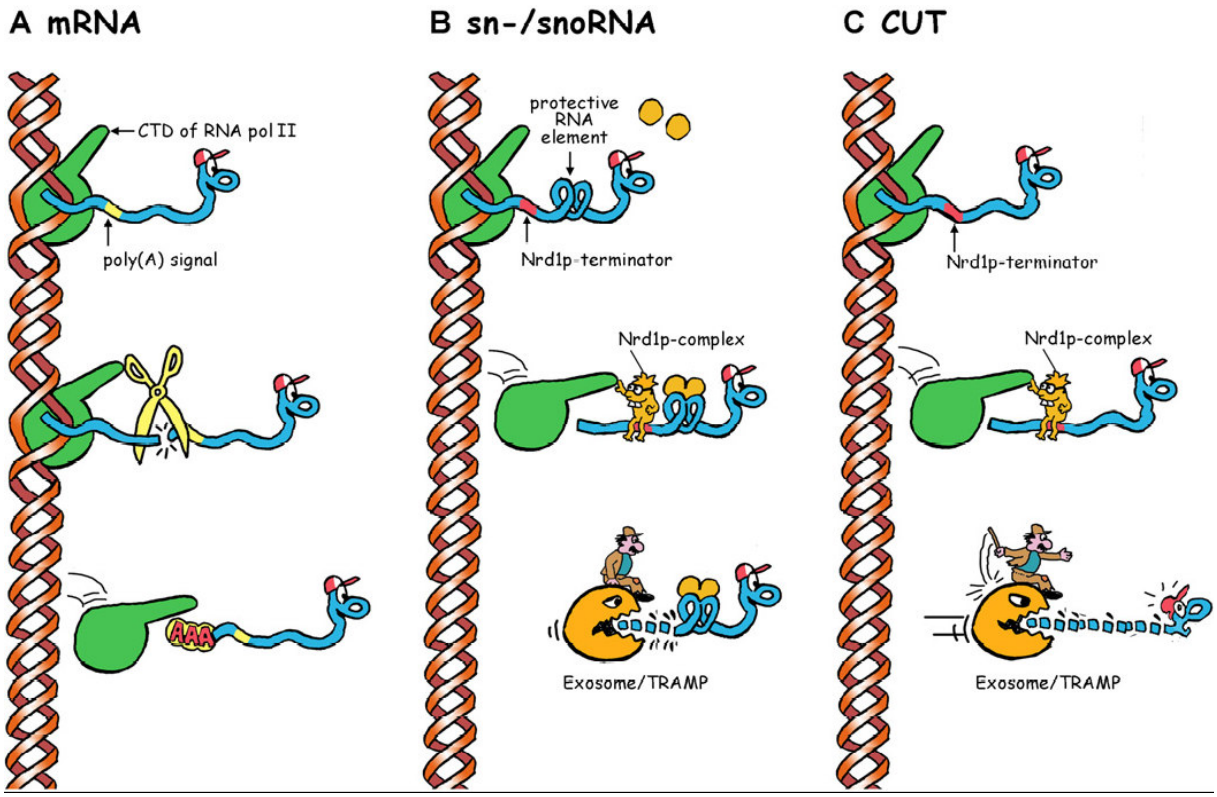

Figure 9 Transcription termination and 3' end processing of three different RNA Pol II derived transcripts (Lykke-Andersen and Jensen, 2007).

2.5 Export of mRNA

The transport of RNA molecules from the nucleus to the cytoplasm is fundamental for gene expression. Different RNA species produced in the nucleus are exported through the nuclear pore complexes via different RNA export pathways (Köhler and Hurt, 2007). Only correctly processed mRNAs are allowed to be exported to the cytoplasm, providing evidence that mRNA maturation and export are linked to each other (Lei and Silver, 2002).

The nascent mRNA is cotranscriptionally coated with proteins and assembled into a messenger ribonucleoprotein (mRNP) by the THO/TREX complex. The THO complex consists of the subunits Tho2p, Hpr1p, Mft1p and Thp2p and forms a robust subcomplex of TREX, which additionally harbours Yra1p, Sub2p and Tex1p (Strässer et al., 2002). The TREX complex physically links proteins that function either in mRNA export or transcription: the THO complex functions in transcription elongation (Chávez et al., 2000), while the subunits Yra1p and Sub2p are required for mRNA export (Strässer et al., 2002), and importantly, all four components of the THO complex interact genetically with both *YRA1* and *SUB2*. Mutants of TREX show an accumulation of poly(A) RNA in the nucleus indicating that also THO mutants have a role in mRNA export. TREX accompanies the elongating RNA Pol II and the THO subcomplex interacts with the chromatin, whereas Sub2p and Yra1p associate with the nascent RNA transcript (Abruzzi et al., 2005). In turn, Yra1p recruits the export receptors Mex67p-Mtr2p, which do not bind the GTPas Ran directly, but interact with nucleoporins and shuttle between nucleus and cytoplasm (Zenklusen and Stutz, 2001) (figure 10).

The nuclear exosome has been implicated in the degradation of nuclear pre-mRNA, unadenylated and 3' unprocessed pre-mRNAs as well as mRNPs assembled with mutant TREX complex (Zenklusen et al., 2002; Bousquet-Antonelli et al., 2000; Hilleren et al., 2001). The exosome is therefore not only a major player in the nuclear mRNA surveillance mechanism that degrades mRNAs with aberrant 3' ends but is also responsible for the degradation of defective mRNPs. How aberrant mRNPs are identified is unknown.

Sac3p was identified as an mRNA export factor having genetic interaction with subunits of the TREX complex and being able to physically recruit the Mex67-Mtr2 export receptor (Fischer et al., 2002; Lei et al., 2003). Sac3p forms together with Thp1p, Sus1p and Cdc31p a complex called Sac3-Thp1-Sus1-Cdc31-complex or TREX-2 (Fischer et al., 2004). This complex is associated with the inner side of the nuclear pore complex via the nucleoporins Nup1p and Nup60p (Fischer et al., 2002). Interestingly, Sub1p is also part of the SAGA complex, a large transcription-initiator that catalysis histone acetylation and deubiquitylation (Shukla et al., 2006; Köhler et al., 2006) suggesting that Sub2p functions in coupling of SAGA-dependent gene expression with mRNA export via its tether to the inner side of the nuclear pore complex (Rodriguez-Navarro et al., 2004) (figure 10).

The model of gene gating was proposed by Günther Blobel and it says that every expressed gene is physically connected to a particular nuclear pore complex in the nuclear membrane (Blobel, 1985). Several recent studies have shown that a number of genes are

dynamically targeted to the nuclear envelope upon activation and this positioning appears to facilitate transcription and subsequent nuclear mRNA export (Taddei et al., 2006; Cabal et al., 2006).

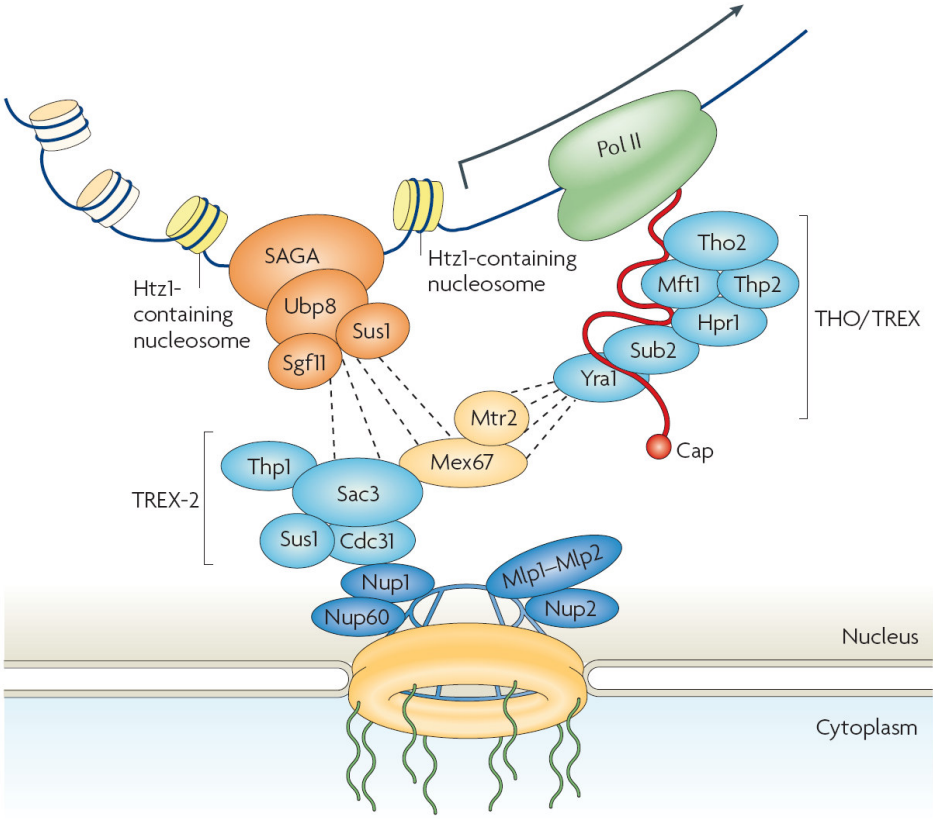

Figure 10 Gene gating and mRNA export (Köhler and Hurt, 2007).

3 Poly(A) polymerase

In eukaryotes, polyadenylation of pre-mRNA plays an essential role in the initiation step of protein synthesis, as well as in the export and stability of mRNAs. Poly(A) polymerase (PAP) is classified as a template-independent polymerase, a category shared only by terminal deoxynucleotidyl transferase. Although it employs the same catalytic mechanism as other nucleic acid polymerases, poly(A) polymerase is significantly different in that it does not utilise a template strand to select and position the incoming nucleotide.

An activity which added adenosine residues to the 3' ends of RNAs was discovered in the early 1960s, and at the time it was a reaction of unknown significance. All eukaryotic genomes code for one or several canonical nuclear PAPs. Yeast has one (Lingner et al., 1991) and higher eukaryotes have two or three copies. In mammals, there exist different forms: PAP I, PAP II, PAP IV, TRAP, neo-PAP and Star-PAP. There has been considerable evolutionary conservation of the amino acid sequence of the N-terminal catalytic domain, with extensive similarities from yeast to human, as yeast and the first 400 nt of mammalian PAP have around 47% identity (Ohnacker et al., 1996).

3.1 Yeast poly(A) polymerase

3.1.1 Canonical poly(A) polymerase

Canonical poly(A) polymerases in eukaryotes are responsible for the addition of poly(A) tails during the processing of the 3' ends of messenger RNA precursors in the nucleus. Yeast Pap1p retains polymerase activity when separated from the holoenzyme assembly and can processively add long stretches of adenosine nucleotides to an RNA primer *in vitro*. Yeast Pap1p was crystallised in combination with two CoTPs, one occupies the position of the incoming base, prior to its addition to the mRNA chain, and the other is believed to occupy the position of the 3' end of the mRNA primer (Bard et al., 2000). Pap1p is composed of three domains that encircle the active site, like it has been observed for other nucleic acid polymerases. In contrast to the incoming CoTP, the mononucleotide primer shows extensive interactions with Pap1p (Bard et al., 2000). Since the enzyme has no contact to the incoming adenosine, no hypothesis for a strict ATP selection can be

made. The C-terminal domain of Pap1p holding an RNA binding domain binds the RNA primer/product.

The association in CPF enhances Pap1p processivity; there is no requirement to induce processivity as in the case of the mammalian PAP (Preker et al., 1997). Fip1p is the only protein shown to interact with Pap1p (Helmling et al., 2001). Fip1p has also been shown to affect the *in vitro* activity of poly(A) polymerase as it inhibits polyadenylation activity of Pap1p. A poly(A)-binding protein is needed to stop the polyadenylation process. First, the major cytoplasmic poly(A)-binding protein Pab1p has been suggested to regulate polyadenylation (Amrani et al., 1997). Then it was thought, that Pap1p recruits and activates the poly(A) nucleases Pan2-Pan3 (Brown and Sachs, 1998), which would imply that a deadenylation step is required for poly(A) tail length control. It has also been proposed that Nab2p is implicated in poly(A) tail lengh regulation (Hector et al., 2002). However, *in vitro* polyadenylation assays, either Pab1p or Nab2p produced the corret poly(A) length of 70 As in the absence of PAN, which was localised to the cytoplasm (Dheur et al., 2005). It seems that in yeast, the process for poly(A) length control is the same as in mammalian cells an does not involve a deadenylation process.

3.1.2 Uncanonical poly(A) polymerases

In yeast, Trf4p and Trf5p have been identified as poly(A) polymerase localised to the nucleus (Vanacova et al., 2005; LaCava et al., 2005). Trf4p and Trf5 are part of the TRAMP complex *in vivo*. The poly(A) tails made by the TRAMP complex primarily serve to initiate RNA degradation (see chapter 2.4.3). Trf4p needs the TRAMP subunit Air1p or Air2 for *in vitro* polyadenylation activity (Vanacova et al., 2005).

3.2 Mammalian poly(A) polymerase

3.2.1 PAP

PAPα was fist identified (Raabe et al., 1991; Wahle et al. 1991) and has also been extensively characterised. The structure of the mammalian PAPα in complex with CoTP has been determined by X-ray crystallography (Martin et al., 2000; Martin et al., 2004).

The crystal structure of the bovine PAP is very similar to the yeast structure, but catalytically important residues of the bovine PAP make specific contacts to the adenine base and discriminate against guanine, cytosine and uracil. PAP strongly discriminates against nucleotides other than ATP (Wahle, 1991; Martin and Keller, 1998) although discrimination against GTP is not as stringent as against pyrimidines. Additionaly, the RNA binding domain of bovine PAP was shown to overlap with the nuclear localization signal and three aspartates essential for catalysis could be identified. The three aspartates ligate two of the three active site metals. One of these metals also contacts the adenine ring (Martin et al., 2000).

In vitro, the interaction of PAP with the CPSF subunits CSPF160 and hFip1p stimulate PAP by tethering it to the RNA for which it has intrinsically a low affinity (Kaufmann et al., 2004; Kerwitz et al., 2003). The poly(A)-binding protein nuclear 1 (PABPN1) interacts with the first synthesised 11 adenosine residues and further increases the affinity of mPAP for the RNA. This activates processive mode of the enzyme (Kerwitz et al., 2003). After approximately 250 A residues have been polymerised in a poly(A) tail, PAP switches back to a distributive mode (Wahle, 1995). The mechanism responsible for this poly(A) tail length control is not fully understood.

In vertebrates, multiple forms of PAP mRNA are synthesized from a single gene by alternative splicing (figure 11). The longest mRNA forms encode PAPs I and II, which are distinguished from each other by their C-terminal sequences (Raabe et al., 1991), whereas competition between splicing and polyadenylation of the mRNA precursor produces short forms of mRNA encoding PAP III, V, and VI (Zhao and Manley, 1996). Of these five PAPs, PAP II is the predominant form in most cells, and contains three functional domains: a catalytic domain, an RNA-binding domain and a large domain rich in serine and threonine at the C-terminus (Martin and Keller, 1996; Martin et al., 1999). PAP I and II also possess two nuclear localization signals, NLS1 and NLS2, which are both required for the efficient transport of the enzymes into the nucleus (Raabe et al., 1994). Functional analysis has shown that the RNA binding domain (RBD) and polymerase module (PM) are essential but not sufficient for catalytic activity. The regulatory Ser/Thr-rich domain provides several consensus and nonconsensus phosphorylation sites (Colgan et al., 1996 and 1998). It is not required for *in vitro* activity and yeast is lacking this domain. The three truncated forms of PAPs III, V, and VI, which terminate in the middle of the protein-coding region of PAP I and II, lack NLS 1 and 2, and Ser/Thr-rich domain. The introns 9, 10 and 12 contain all an AAUAAA hexanucleotide (Zhao and Manley, 1996). Although

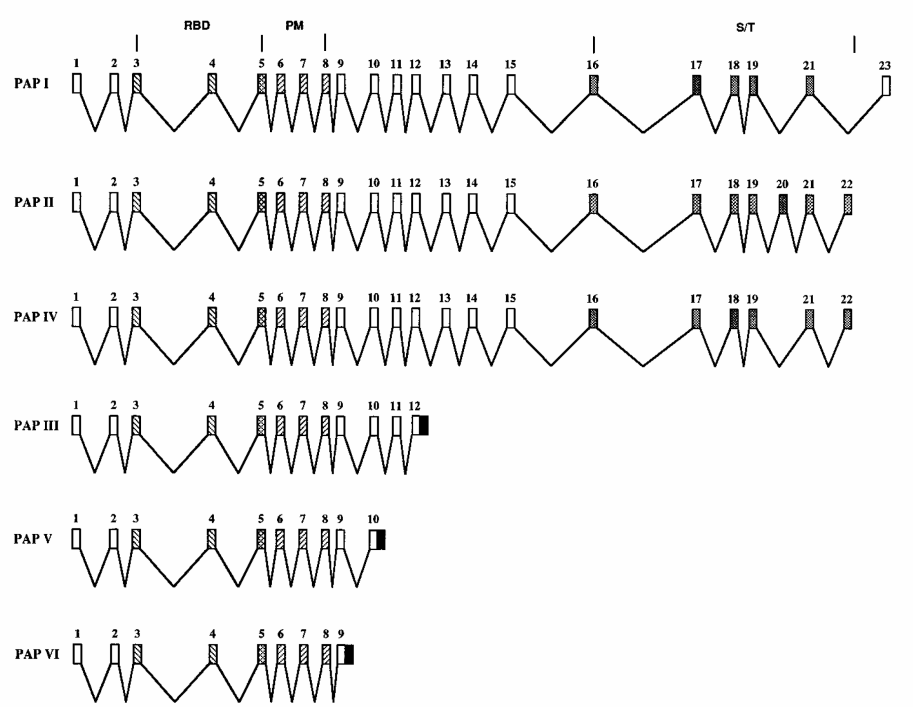

Figure 11 Structures of the alternatively spliced forms of PAP. (Zhao and Manley, 1996)

The numbered open boxes display the exons, and splicing is indicated by angled lines between exons. The RNA binding domain (RBD) is located in exons 3, 4 and 5, the polymerase module (PM) in exons 5, 6 and 7, NLS1 in exon 16, NLS2 in exon 19 and Ser/Thr-rich region (S/T) is located from exon 16 to 22.

the mRNAs for PAP III, V, and VI are indeed detectable in cells, the proteins translated are below the limit of detection, and the recombinant proteins have no polyadenylation activity (Thuresson et al., 1994; Zhao and Manley, 1996). Thus, the functions of these three PAP are unknown.

During cell cycle, PAP has been shown to carry different modification patterns. Mitosis/meiosis-promoting factor (MPF) is a heterodimeric cyclin-dependent kinase consisting of p34cdc2 and cyclin B and phosphorylates a wide range of targets, acting as a 'master regulator' of mitosis (King et al., 1994). PAP is also phosphorylated by MPF at totally seven consensus sites (Colgan et al., 1996) and non-consensus sites (Colgan et al., 1998). Direct phosphorylation assays *in vitro* revealed that phosphorylated PAP showed a significant reduction in activity, which could be reversed by phosphatase addition. Phosphorylation of all seven phosphorylation sites results in an nearly total repression of activity, while partial phosphorylation has no detectable effect. This modification has been

demonstrated for PAP I and PAP II. Yeast Pap1p lacks the C-terminal Ser/Thr rich domain which might suggest that cell cycle regulation of PAP activity might be restricted to metazoa.

3.2.2 TRAP

TRAP was identified as a testis-specific poly(A) polymerase in mice, that is localized to the cytoplasm of germ cells (Kashiwabara et al., 2000). TRAP gene is a single copy gene on the genome and is therefore not splicing variant derived from the PAP gene. The fact that TRAP is expressed in spermatogenic cells, could imply that additional poly(A) extensions of existing mRNAs might be added by TRAP in the cytoplasm of these cells, maybe as a step of post-transcriptional regulation. However, the function of TRAP is still not fully understood.

3.2.3 Neo-PAP

Neo-PAP was identified as a form of PAP by molecular cloning from a human tumor cell cDNA library (Topalian et al., 2001). Due to its identification and overexpression in human neoplasms, this molecule was named neo-poly(A) polymerase (neo-PAP). Intriguingly, the intron-exon organization of the PAP and neo-PAP genes is almost identical, suggesting that they arose by gene duplication and subsequent recombination. Neo-PAP is indistinguishable in its biochemical functions *in vitro* from PAP. However, significant sequence dissimilarities in the C-terminal domain of neo-PAP and PAP as well as apparent differences in the phosphorylation of these two molecules suggest that each may be influenced by distinct regulatory controls. Neo-PAP is not phosphorylated throughout cell cycle although it provides consensus phosphorylation sites. Neo-PAP may therefore be an important RNA processing enzyme that is regulated by a mechanism distinct from that utilized by PAP.

3.2.4 Star PAP

Star PAP (nuclear speckle targeted PIPKI regulated poly(A) polymerase) was identified as a type I phosphatidylinositol 4-phosphate 5 kinase (PIPKI) interacting protein (Mellman et al., 2008). PIPKI localises to nuclear speckles and Star-PAP colocalises with PIPKI. It is unique among known PAPs, because it contains a catalytic domain splitted by a praline-rich region. In addition, it contains a zinc-finger and only one NLS. Poly(A) polymerase activity of Star-PAP is stimulated *in vitro* by the presence of phosphatidylinositol-4,5-biphosphate (Ptdlns-4,5P_2) that is generated by PIPKI (Heck et al., 2007). Othe rphosphoinosities did not affect Star-PAP activity, and no phosphoinositide had an effect on PAP I. Star-PAP was associated with HO-1 mRNA and was required for efficient 3' processing. HO-1 is an important cytoprotective enzyme for reactive oxygen induced stress response (Duckers et al., 2001). It was speculated that antioxidant response might induce the assembly of a Star-PAP complex together with PIPKI. PIPKI would produce Ptdlns-4,5P_2 that controls processivity of Star-PAP.

VI Results

1 Molecular effects of cordycepin in *Saccharomyces cerevisiae*

Cordycepin as an adenosine analogue without the 3' OH group is thought to inhibit the process of polyadenylation in eukaryotic cells by preventing the addition of further adenosines after it's incorporation into the poly(A) tail (Butler et al., 1990), especially at lower concentrations (Zeevi et al., 1981). This compound is also widely used in experiments as a polyadenylation inhibitor *in vitro* as well as *in vivo* (Ioannidis et al., 1999; Costanzo et al., 2001; Gershon et al., 2006). The aim of this project is to characterise the molecular effects of cordycepin particular the effect RNA metabolism in the yeast *Saccharomyces cerevisiae*.

In early studies, it was suggested that *S. cerevisiae* is resistant to cordycepin (Anderson and Roth, 1974). Later, it was found that *S. cerevisiae* cultivated in thiamine-free medium is sensitive to cordycepin and that the presence of thiamine increases growth resistance for the drug (Iwashima et al. 1992). Iwashima and co-workers found two mutant strains, which are resistant to the inhibitory action of cordycepin: CD-R1 was found to be deficient in a common transport system for thiamine and therefore cordycepin might not been taken up by the cells. The CD-R2 mutant had no defect in the thiamine import system, but the incorporation of cordycepin into RNA was markedly lower suggesting that the conversion of cordycepin into a more active form is impaired in this mutant. Later, a lesion in adenosine kinase was identified in the mutant strain CD-R2 (Iwashima et al., 1995).

According to that, cordycepin might be taken up by the cells via the thiamine transport system, as the yeast *S. cerevisiae* does not take up exogenous adenosine and also can not use it as a purine source (Mäser et al., 1999). Cordycepin is then converted to cordycepin monophospate (CoMP) by the enzyme adenosine kinase (*ADO1*) (Lecoq et al., 2001), which has a major role in recycling adenosine produced from S-adenosyl-methionine (AdoMet) by the methyl cycle (Barrado et al., 2003). *ADO1* is not an essential gene, because in the absence of *ADO1*, AMP can still be produced using the purine *de novo* biosynthesis system or xanthine as a source. CoMP is transformed into the active form CoTP by the enzyme adenylate kinase (*ADK1*) (Konrad, 1988). Adk1p is not

essential, probably because of the existence of a second adenylate kinase, Adk2p, normally localised to the mitochondria (Cooper and Friedberg, 1992).

1.1 Mutant strains of factors involved in 3' end formation are cordycepin sensitive

Since cordycepin acts as a chain terminator of transcription elongation and polyadenylation, its presence should lead to a higher growth impact on strains carrying mutations affecting 3' end formation. Mutation and cordycepin could exhibit a synergistic effect. This was tested with a drop test, where dilutions of yeast cultures were dropped on plates either containing or lacking cordycepin (figure 12). The addition of 40 µg/ml cordycepin does only cause a very slight growth defect in wild-type strains, but several

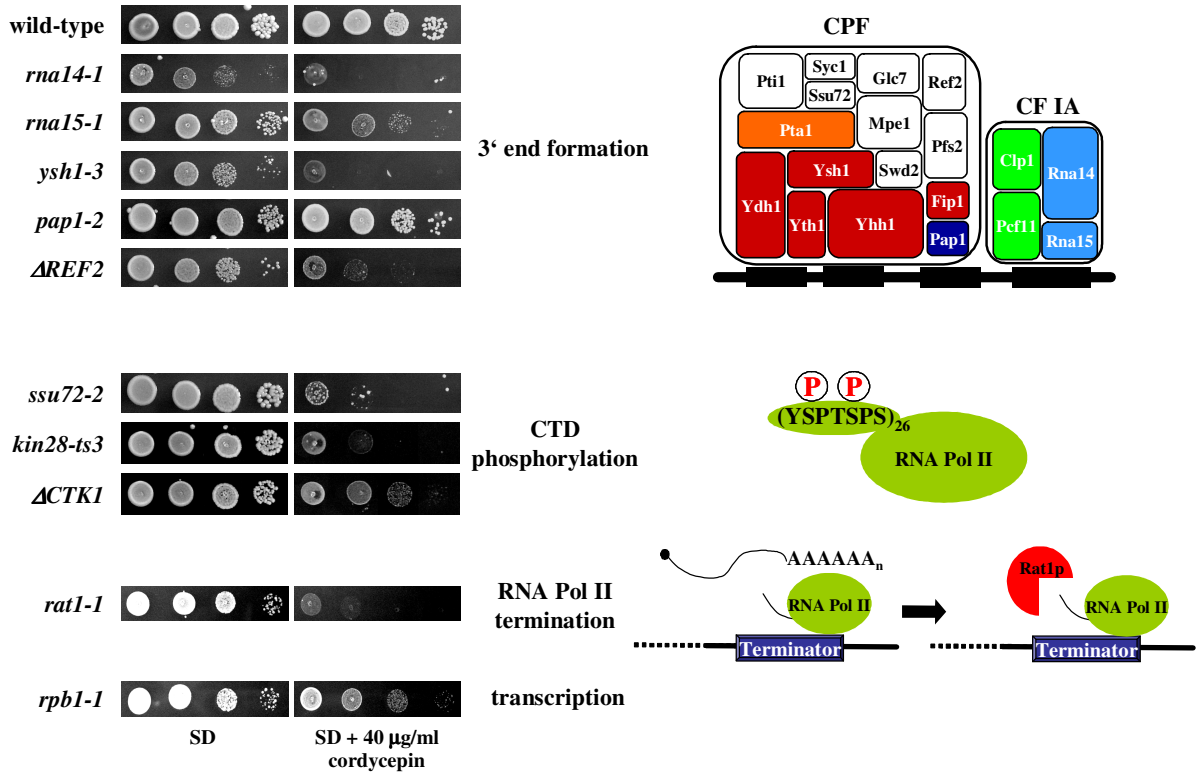

Figure 12 Mutation of factors involved in 3' end formation causes a growth inhibition in presence of cordycepin.

Ten-fold dilutions of yeast strains indicated at the left were dropped on SD plates containing or lacking cordycepin [40 µg/ml] and incubated at 25 °C for 3 days. Cleavage and polyadenylation factor (CPF) and cleavage and polyadenylation factor IA (CP IA), the phosphoryated CTD and transcription termination are illustrated on the right.

mutant strains showed a severe growth phenotype in presence of cordycepin. Mutants of *RNA14* and *RNA15* were initially identified as cordycepin (Bloch et al., 1978) and both encode for factors of the cleavage and polyadenylation factor (CF IA). The mutant strains *rna14-1* and *rna15-1* show defects in cleavage and polyadenylation demonstrating their role in 3' end formation. Both mutant strains showed a strong sensitivity against the drug cordycepin, as growth is strongly inhibited in presence of cordycepin compared to that on normal media. Mutation of *YSH1*, which is thought to be the endonuclease, that cleaves the nascent RNA transcript (Chanfreau et al., 1996), displayed as well strong cordycepin sensitivity. A nonessential factor involved of this machinery is Ref2p, which also shows a clear growth inhibition in presence of the adenosine analogue. As well factors affecting the phosphorylation status of the CTD were highly sensitive to cordycepin. Ssu72p exhibits a phosphatase activity for Ser 5, Kin28p is the Ser 5 kinase and CTK1 holds a kinase activity for Ser 2. Mutations affecting either the process of transcription (*rpb1-1*) or transcription termination (*rat1-1*) were also sensitive to cordycepin.

1.2 Mutation of *PAP1* leads to cordycepin resistance

Most interestingly, mutation in the *PAP1* gene revealed cordycepin resistance. This observation was highly unexpected, since Pap1p is the enzyme synthesising the poly(A) tail on the pre-mRNA. This is exactly the process, which is thought to be affected by cordycepin. Different temperature-sensitive mutant alleles of *PAP1* were tested and all seemed to be resistant to cordycepin (figure 13). Interestingly, the mutant strains *pap1-2*, *pap1-5* and *pap1-7* appeared to grow even slightly better at semi-permissive temperature (33 °C) in presence of cordycepin than without.

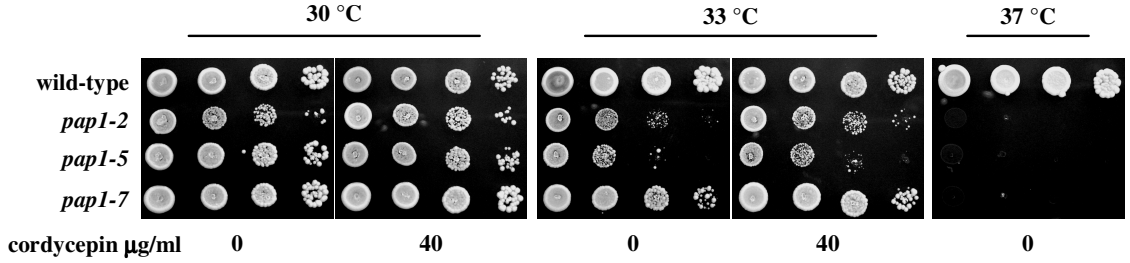

Figure 13 *PAP1* temperature sensitive mutant strains are resistant to cordycepin.

Ten-fold serial dilutions of the yeast strain indicated on the left were dropped on SD plates containing or lacking cordycepin [40 µg/ml]. The plates were incubated at the indicated temperatures for 3 days.

1.3 Effect of cordycepin on poly(A) tails

Since cordycepin is thought to have an affect on polyadenylation, poly(A) tail length distribution was analysed in wild-type cells in presence of cordycepin. Wild-type cells shifted to 40 µg/ml cordycepin showed a decrease in the poly(A) tails levels after 60 minutes (figure 14 A). The poly(A) tail signal was even fainter after 120 minutes. Poly(A) tails seems to decrease in presence of cordycepin. When wild-type cells were grown for 6 hours at different cordycepin concentrations, the signal for the poly(A) tails was as well decreasing (figure 14 B). The addition of 20 – 60 µg/ml cordycepin resulted in a slight decrease of poly(A) levels, 80 µg/ml cordycepin led to a pronounced decrease of poly(A) tails. However, the length distribution of the poly(A) tails did not change upon cordycepin addition.

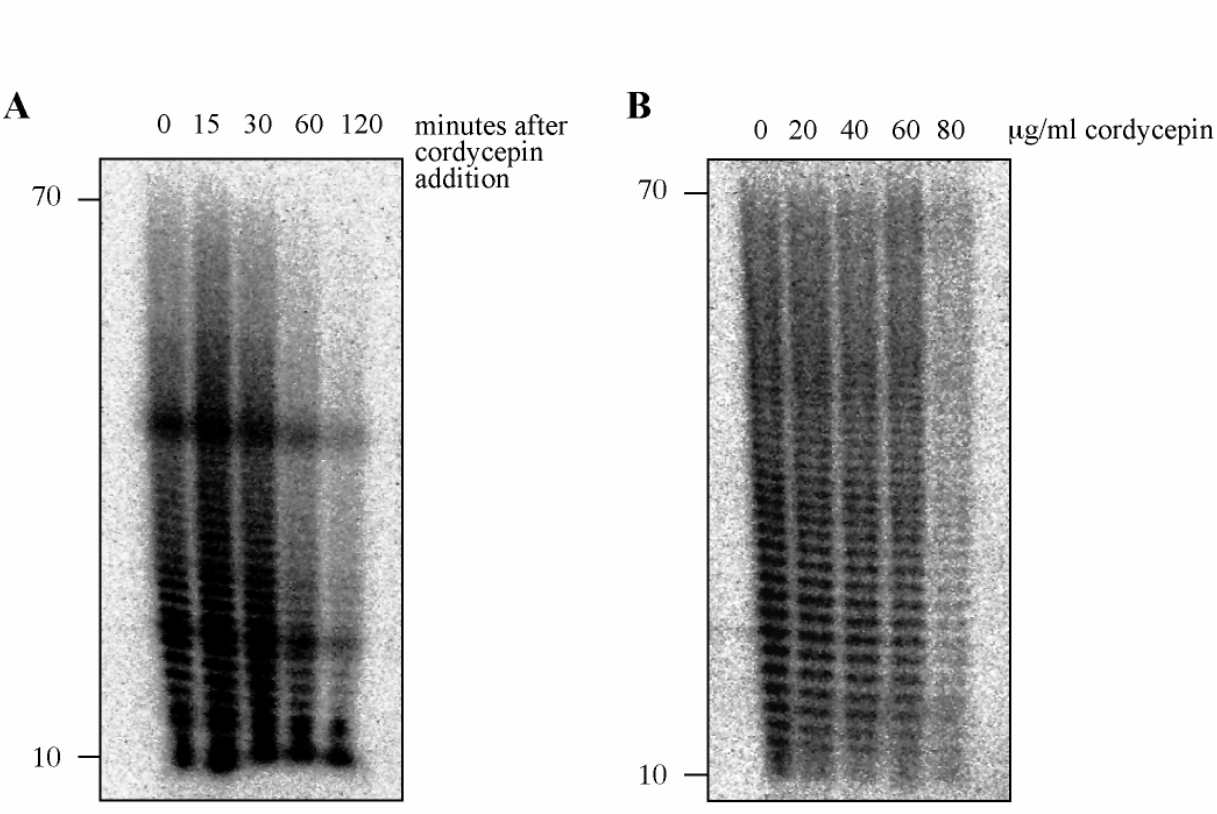

Figure 14 Poly(A) tail length distribution analysis.

Poly(A) tail length distribution assay of wild-type cells. Total RNA was extracted and labelled with Pap1p and radioactive cordycepin. The RNA was digested with RNase A and T1 and the remaining poly(A)RNA was separated on a 15% polyacrylamid gel. The position and size in number of nucleotides is indicated on the left. ***A)*** *Wild-type cells were shifted to 40 µg/ml cordycepin and cells were harvested at the indicated time points.* ***B)*** *Cells were cultured at the indicated cordycepin concentration and harvested after 6 hours.*

1.4 Cordycepin affects poly(A) site selection

RNA levels were examined in wild-type strains after a shift to cordycepin (figure 15). A decrease in mRNA levels of *CYH2*, *ADH1*, *ACT1* and *ASC1* was observed, with a pronounced drop between 60 min and 120 min. A reduction of RNA levels has as well been observed for transcriptional inhibitors such as 6-Azauracil (Lennon et al., 1998) or

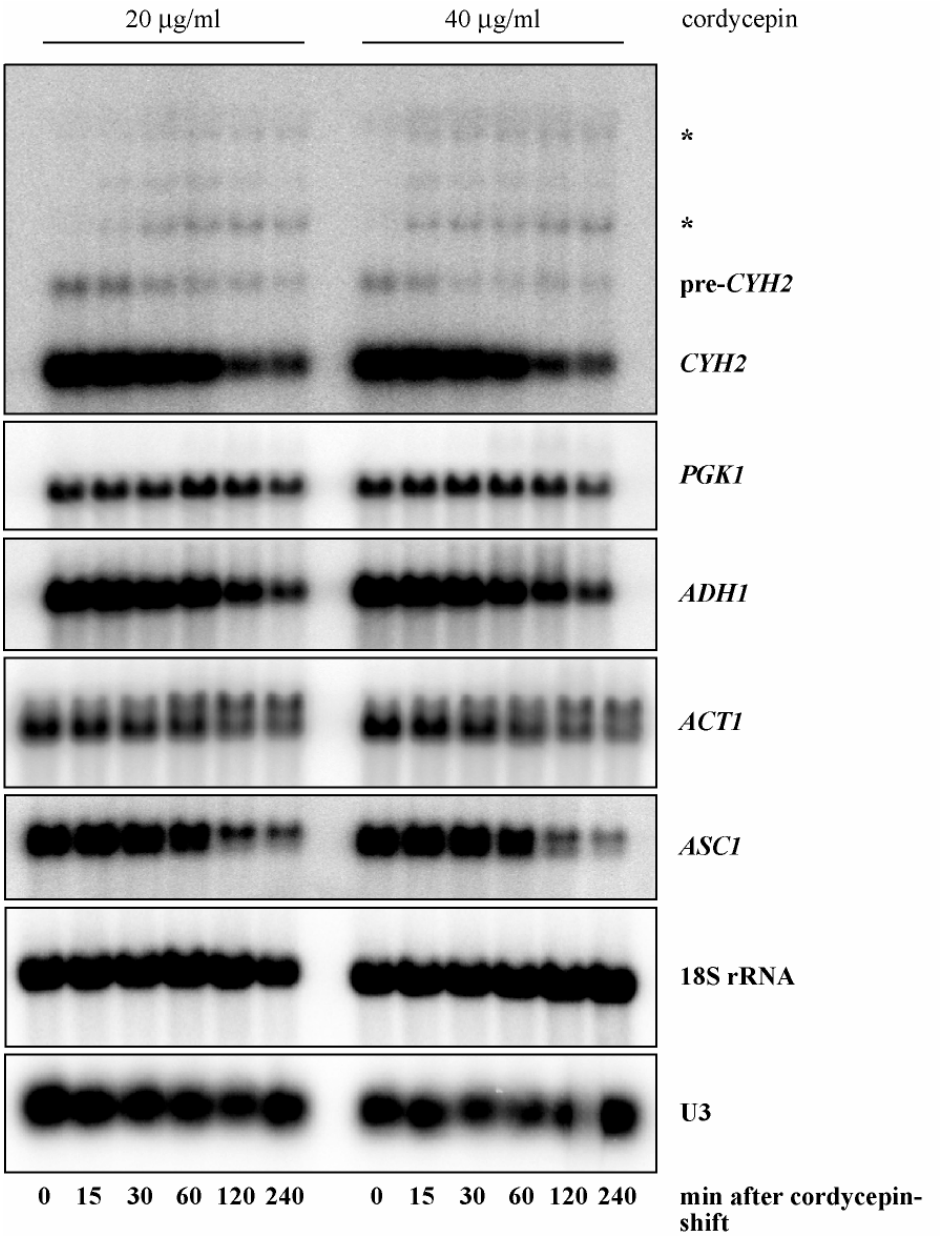

Figure 15 RNA levels after the addition of either 20 or 40 µg/ml cordycepin.

Northern blot of total RNA extracted from wild-type cells at the indicated time after the shift to media containing cordycepin at the displayed concentration. The membrane was hybridised with random-primed labelled probes against the different mRNA species and with labelled primers against 18 S rRNA and U3. The asterix marks extended RNAs.

cells carrying a temperature sensitive mutation in *RPB1* (Herrick et al., 1990). The levels of *PGK1* mRNA were only slightly affected by cordycepin, because *PGK1* is a very stable mRNA with a half-time of more than 90 minutes (Wang et al., 2002). The observed level of the decrease might be related to the respective mRNA stability. The RNA levels of 18S rRNA and U3 were as well not affected by cordycepin. The same effect was observed with a drug concentration of 40 µg/ml. *CYH2*, *ASC1* and *ACT1* pre-mRNA contain introns, but only the pre-mRNA of *CYH2* can be detected on a Northern blot. Furthermore, additional longer *CYH2* transcripts could as well be observed. To test, whether the extended *CYH2* RNAs still contain the intron, the membrane was hybridised with a probe against the intron sequence of *CYH2* (figure 16). The extended RNAs were detectable with an intron-probe indicating that these RNAs were unspliced read-through products.

Extended RNAs were also observed for *ACT1* and *ASC1* transcripts, although the enlarged RNAs were similar in length leading to the suggestion, that different poly(A) sites may be used to generate these extended transcripts. To analyse the distribution of the 3' ends of the *ASC1* and *ACT1* transcripts at different time points after the shift to cordycepin, the RNAs were analysed on an acrylamide Northern gel after cleavage with RNase H and an oligonucleotide near the 3' end of the open reading frame. In the wild-type strain, the *ACT1* mRNA has five 3' ends (Manhart and Parker, 1995) labelled I to V on the scheme of the 3' end of *ACT1* (figure 17 A). Under normal growth conditions, site I

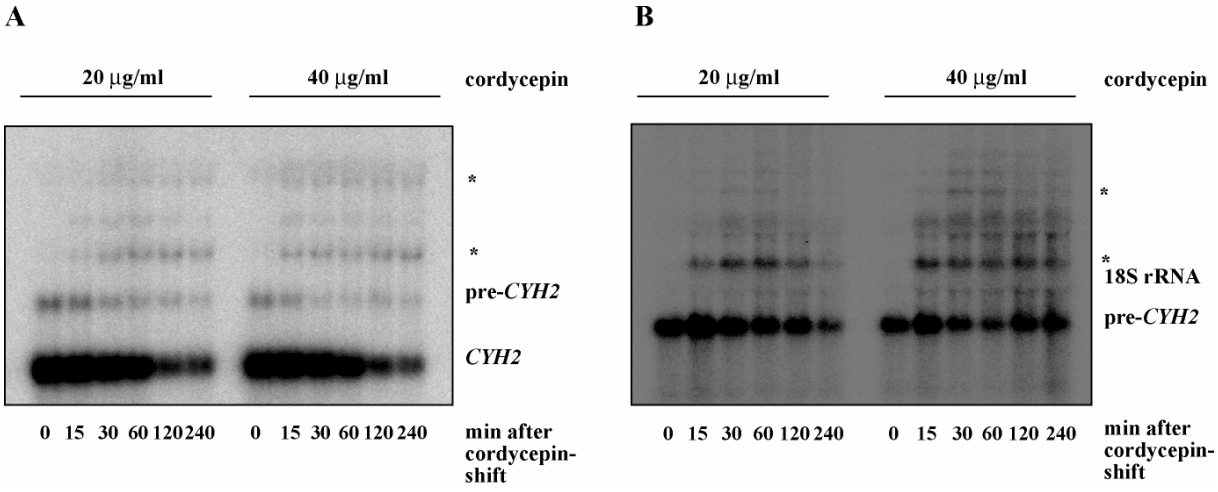

Figure 16 RNA levels for *CYH2* mRNA and pre-mRNA.

*Northern blot of total RNA extracted from wild-type cells at the indicated timepoints after the shift to media containing cordycepin at the displayed concentration. The membrane was hybridised with random-primed labelled probes against **A)** CYH2 mRNA and **B)** CYH2 intron. The asterix marks extended RNAs.*

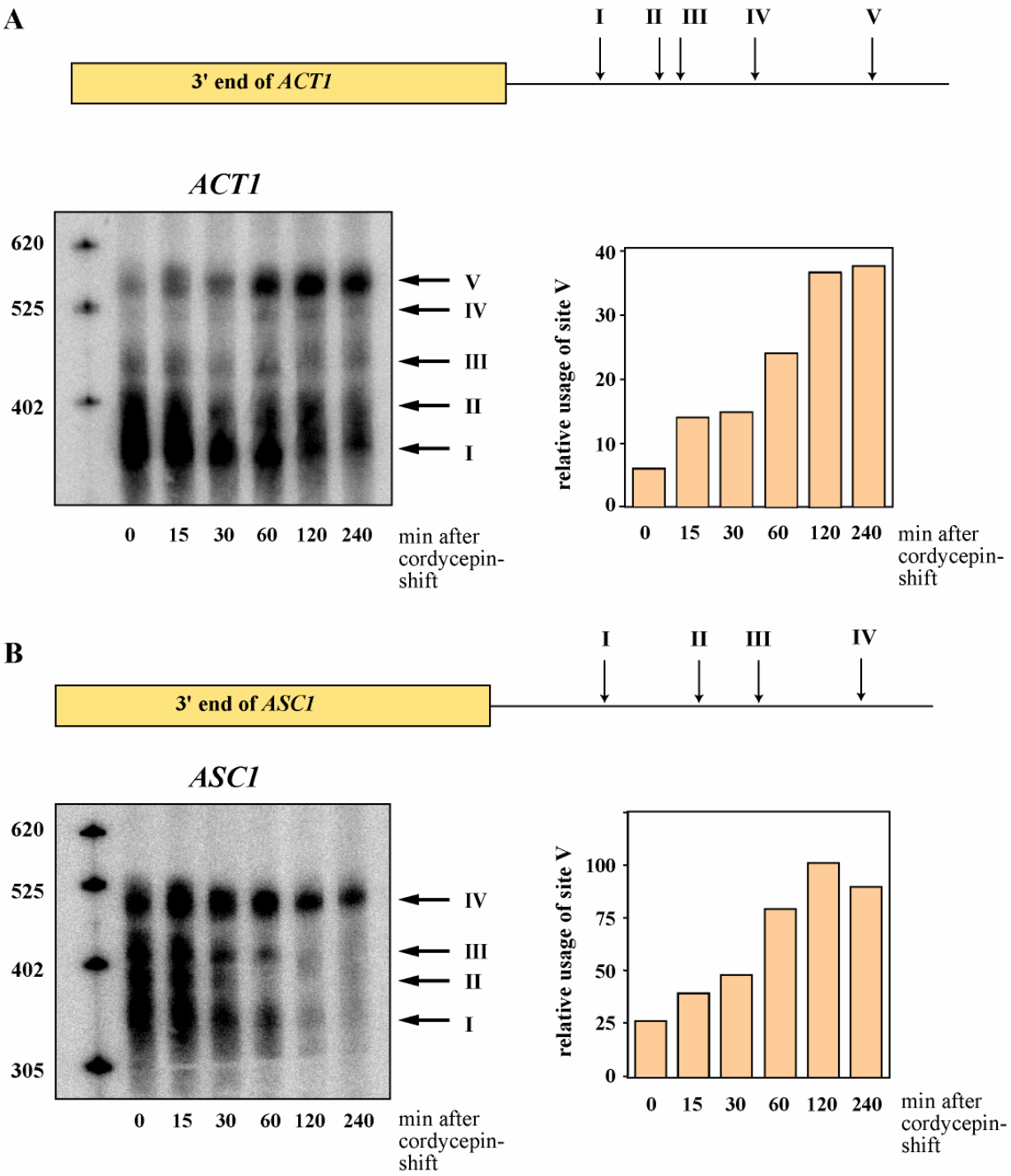

Figure 17 Cordycepin alters the relative levels of different 3' ends of the *ACT1* and *ASC1* mRNA.

A) *Total RNA extracted from wild-type cells was digested with RNase H at the 3' end of the open reading frame, separated by a 8 % denaturing polyacrylamidgel and blotted to a nitrocellulose membrane. The membrane was hybridised with random-primed labelled probes against the 3' UTR to analyse the relative usage of the different poly(A) sites. A scheme of the observed poly(A) sites is shown at the top and the quantification of the relative usage of the most distal site is shown on the right.* ***B)*** *The same analysis for ASC1.*

is used the most and almost 60% of the *ACT1* transcripts are cleaved at site I (Manhart and Parker, 1995), whereas site V is used only to 11%. However, the relative usage of the different poly(A) sites changed dramatically in the presence of cordycepin. The same five poly(A) sites were detectable, but the relative usage of site V changed from 6% to almost 40% after 120 minutes after the cordycepin shift.

Similar results were also seen with the *ASC1* mRNA. However, before the addition of cordycepin four poly(A) sites were detectable labelled I to IV (figure 17 B). The relative usage of the most distal poly(A) site IV is 25%. This proportion changed to almost 100% after 120 minutes after cordycepin addition. These results suggest that cordycepin leads to an alteration in the specificity of the site of polyadenylation. Two possible explanations could interpret that cordycepin led to the production of different levels of the multiple 3' ends of *ACT1* and *ASC1* mRNAs. On the one hand, cordycepin may affect the relative stabilisation of the different transcripts by either destabilising the shorter transcripts or by stabilising the longer mRNAs. On the other hand, cordycepin could interfere with the poly(A) site recognition mechanism by altering the actual efficiencies of the different polyadenylation sites.

1.5 Concentration dependent effect of cordycepin

The RNA levels of different RNA species were analysed in wild-type cells at different time points after a shift to different concentrations of cordycepin. Levels of 18 S rRNA and $tRNA_3^{LEU}$ were not affected by cordycepin, not even at the highest used concentration of 96 µg/ml (figure 18). In the case of mRNAs, the decrease of the respective mRNA signal was generally more prominent at the highest concentration. The decrease is again mRNA dependent, as the decrease in *ADH1* and *ACT1* mRNA levels was more dramatic than for the stable *PGK1* mRNA, which seemed to be resistant to the presence cordycepin for at least 120 minutes.

However, the extended RNAs of *CYH2* and the poly(A) site shift of *ACT1* and *ASC1* mRNAs seemed to be produced most efficiently at a cordycepin concentration of 16 µg/ml. This is most apparent in the case of ASC1 by comparing the signal at 120 minutes after the shift to cordycepin at different concentrations. 16 µg/ml of cordycepin caused an almost complete change in the poly(A) site usage to the most distal site. At a

concentration of 32 µg/ml the relative usage of site IV is reduced and at 96 µg/ml the lower band seemed to be as intense and the upper band. These results suggest, that cordycepin has a concentration dependent effect. At lower concentrations, the drug might

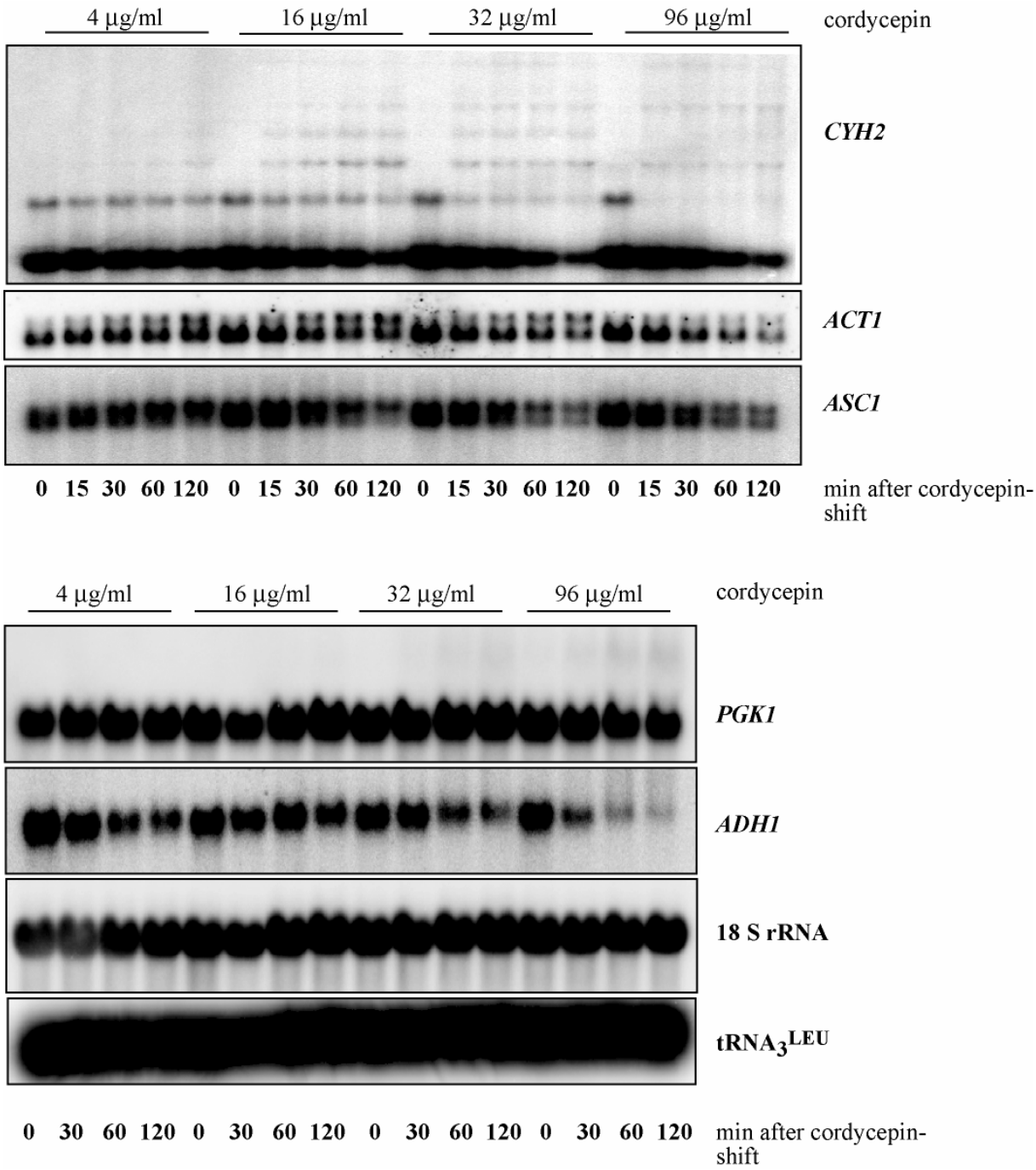

Figure 18 Cordycepin effects mRNA in a concentration dependent manner.

Northern blot of total RNA extracted from wild-type cells at the indicated time after the shift to cordycepin at the indicated concentration. The membrane was hybridized with random-primed labelled probes against the different mRNA species and with labelled primers against 18S rRNA and $tRNA_3^{LEU}$.

interfere with the mechanism of polyadenylation site recognition leading to an increase in the use of the more distal poly(A) site. At higher concentration the primary effect of cordycepin might be the interference with the process of transcription *per se* leading to a drop of total mRNA levels. 18S rRNA and $tRNA_3^{LEU}$ levels were not changed even after the addition of hight concentration of the drug.

1.6 Mutations in *PAP1* suppress cordycepin induced changes in poly(A) site usage

The CF IA subunits *RNA14* and *RNA15* are involved in polyadenylation site recognition, as mutations in both genes alter poly(A) site usage in favour for the most distal polyadenylation site (Mardart and Parker, 1995). A similar poly(A) site shift has been reported for the *pap1-1* mutant strain. Since mutations in these three genes cause a poly(A) site shift, the distribution of the 3' ends of *ACT1* was analysed in presence of cordycepin. An alteration in the distribution of the polyadenylation site usage was observed in the *rna14-1* mutant strains after the addition of cordycepin at 30 °C and as well at 33 °C (figure 19). However, all tested *PAP1* mutant strains do not show any change in the poly(A) site usage in presence of cordycepin at permissive, semi-permissive or restrictive temperature. Cordycepin is therefore able to act differently in mutant strains with altered poly(A) site distribution. This result could indicate that cordycepin resistance correlates with the suppression of a poly(A) site shift on *ACT1* mRNA (see below).

1.7 Microarray analysis of wild-type and *pap1-1* in absence and presence of cordycepin

Cordycepin caused an alteration in the polyadenylation site usage in *ACT1* and *ASC1* mRNA in wild-type cells, but not in the cordycepin resistant *PAP1* mutant strains. How cordycepin changes polyadenylation site usage is unclear, as well as the mechanism by which the resistant *PAP1* mutants suppresses the actions of cordycepin. To get further insight into the primary effects of cordycepin and the mechanism by which cordycepin could interfere with poly(A) site recognition, gene expression levels was analysed by

microarray. In such a gene expression profiling experiment, the levels of thousands of transcripts can be simultaneously monitored to study the effects of treatments such as the addition of cordycepin. With this tool genes can be identified whose expression is changed in response to cordycepin. This might provide significant insight into the biological mode of action of cordycepin.

The outline of this experiment was to compare RNA levels of wild-type strain S288C and the temperature sensitive mutant strains *pap1-1* in presence and absence of cordycepin. Both strains were cultivated at 25 °C and shifted to 40 µg/ml cordycepin for 60 minutes; the *pap1-1* mutant strains was furthermore shifted to restrictive temperature without cordycepin to analyse whether the loss of *PAP1* and the addition of the *PAP1*

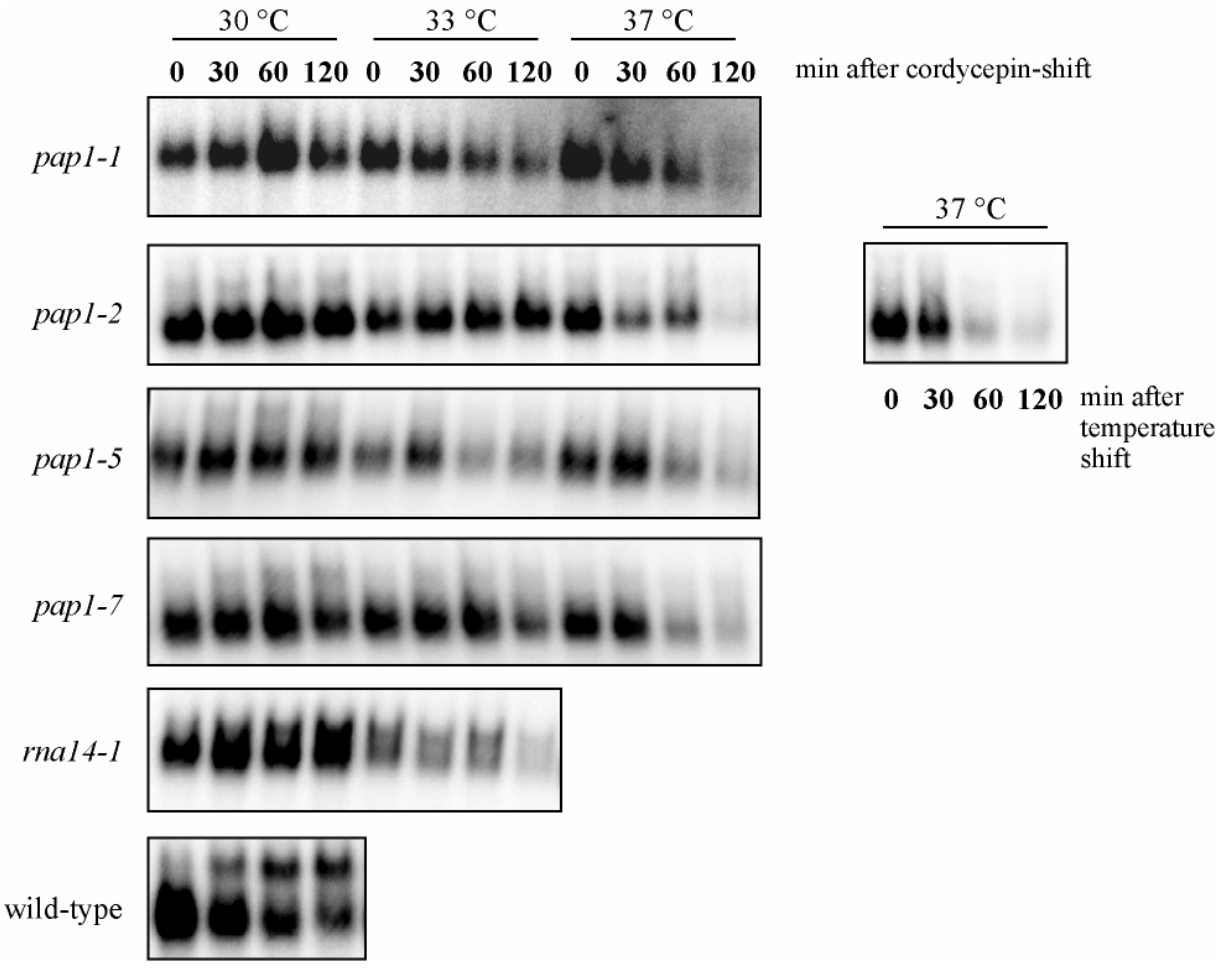

Figure 19 Cordycepin suppresses poly(A) site shift in *PAP1* mutant strains.

Northern blot of total RNA extracted from indicated strains at the indicated time after the shift to [40 µg/ml] cordycepin at the indicated temperature. The membrane was hybridised with random-primed labelled probes against ACT1 mRNA.

inhibitor cordycepin indeed initiate the same response in the cell. All cultures were grown in triplicates and all four conditions were analysed relative to the untreated wild-type to directly compare all four conditions. Total RNA was extracted and reverse transcribed into cDNA using an oligo(dT) primer and a random nonamer primer. For the reverse transcription reaction, aminoallyl dUTP was provided as a component of the deoxynucleotide stock. Incorporated aminoallyl dUTP was coupled with the N-hydroxysuccinimid linked flourophorr Cye3 or Cye5. The fluoroscent targets were pooled and hybridised under stringent conditions to the oligo array. Laser excitation of the incorporated targets yielded an emission with a characteristic spectrum, which was measured by a scanning confocal laser microscope (figure 20). The resulting monochrome images were imported into software (GenePix) in which the images were pseudo-coloured and merged. Data from each single hybridisation dot was expressed as the $\log_2$ scale of the normalised ratio of Cye3/Cye5. According to this, a $\log_2$ value of 0 stands for equality in the respective gene expression and positive numbers indicate a relative increase in the

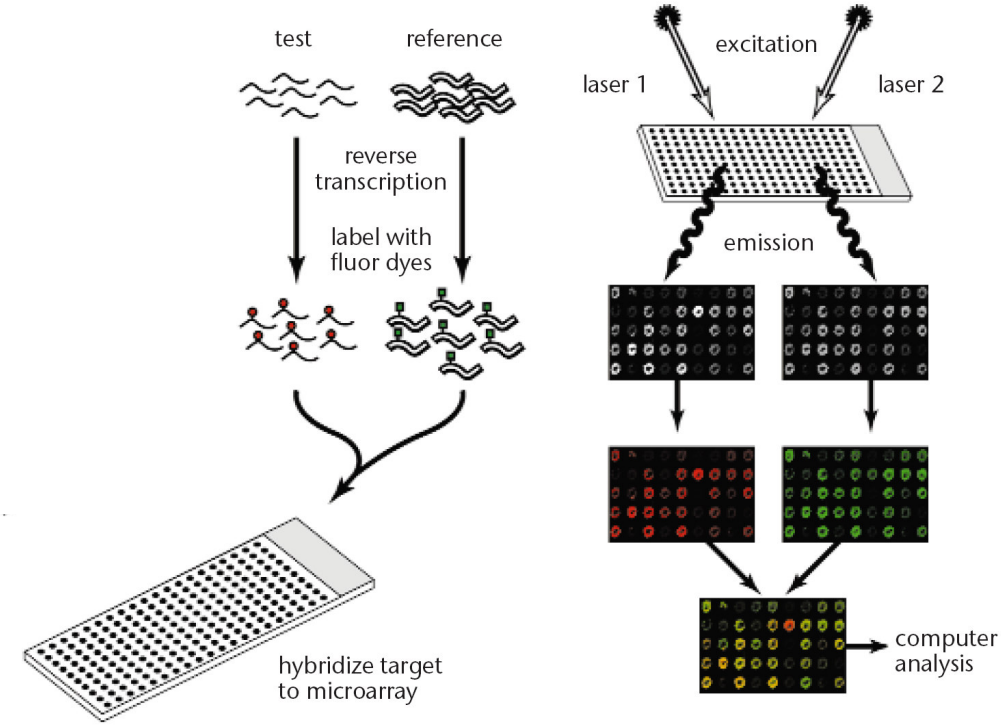

Figure 20 Graphical representation of the different steps of a two-colour microarray experiment (modified from Duggan et al., 1999)**.**

respective RNA abundance and negative numbers represent a decrease relative to the reference. For this experiment, the limit for increased and decreased RNA levels was set at a $\log_2$ value of 1 of -1 respectively; meaning when the respective RNA levels were at least two-fold increased or decreased.

1.7.1 Effect of cordycepin in the wild-type strain

The data of two individual experiments were collected, combined and the average for each data point was calculated. This average number was used for further analysis. Cordycepin caused in the wild-type strain an increase fold in 222 RNAs, and a decrease in 72 RNAs. This is a surprising result since one could assume that cordycepin as a inhibitor of polyadenylation might cause a more general decrease of RNA levels. All identified increased or decreased RNAs were categorised with the Gene Ontology (GO) terminology (figure 21). The cellular compartment with the highest number of proteins generated of the increased RNA section were the nucleus (80 proteins), followed by the cytoplasm (39 proteins), mitochondrion (24 proteins), cytosol (8 proteins) and the endoplasmatic reticulum (7 proteins), however, most proteins (93) could no be localised to a specific compartment. The cellular functions allotted with the highest number of proteins were: catalytic function (45 proteins), transport (11 proteins) and transcriptional regulation (5 proteins). The majority of proteins (129) however, could not be assigned to a specific cellular function. The three most prevalent biological processes were a cellular process (98 proteins), metabolic process (54 proteins) and transport (23 proteins), but for most proteins (102), the biological process was not known.

The decreased RNA section was examined the same way and most of the respective proteins localise to the nucleus (37 proteins), followed by the cytoplasm (25 proteins), the mitochondrion (13 proteins), the cytosol (11 proteins) and for 37 proteins the cellular localisation is not known. 27 proteins hold a catalytic activity, 7 proteins a transporter activity and 31 could not be assigned to a specific cellular function. Cordycepin affected RNAs involved in all kind of cellular compartments, and similarly affected different biological processes and molecular functions. But none of the gene ontology terms was significantly overrepresented in the increased or the decreased RNA cluster. Cordycepin seemed to exhibit a more uniform cellular effect since no biological pathway was activated

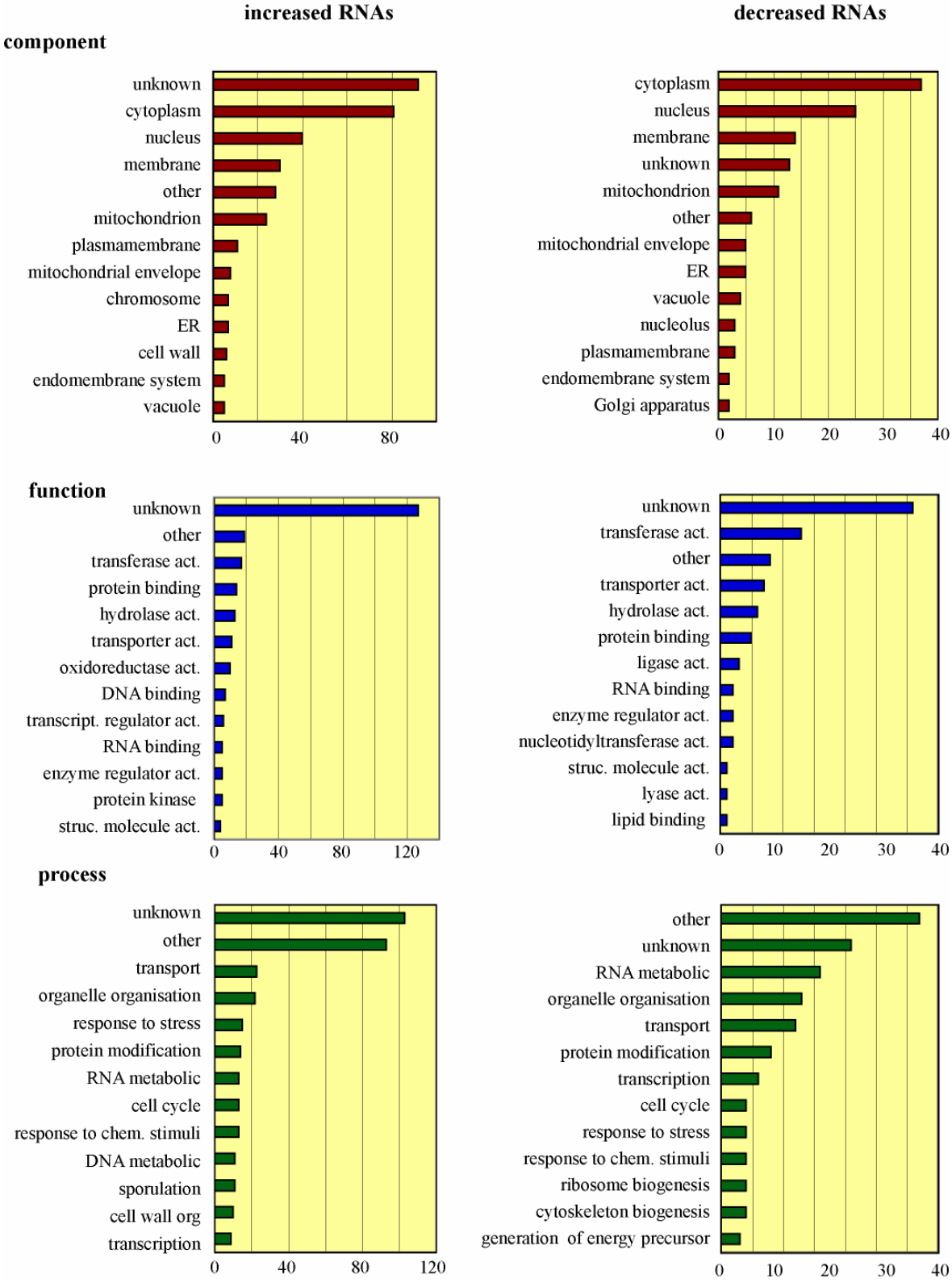

Figure 21 Cordycepin seems to stabilise RNA transcripts derived from hORFs.

Increased and decreased RNA in wild-type strains after cordycepin addition were analysed according to the annotated compartment, function and process of the respective protein. The numbers reflect the amount of RNAs annotated to the specific term.

or inactivated in response to the presence of the drug, which makes it difficult to get further evidence in the characterisation of the mode of action of this compound.

An interesting observation was that the analysis of the 222 increased RNAs revealed that the greatest section of these RNAs could not be annotated to any compartment, function or process (figure 21). The amount of RNA transcripts of unknown function seemed to accumulate in presence of cordycepin. 44.1% of the increased RNA transcripts were annotated to the term hypothetical ORF (hORF), whereas only 27.4% of the decreased RNA fraction and 22.6% of the number of RNAs obtained after processing the raw data were hORFs. Generally, hORFs describe open reading frames of unknown function or genes for which only extremely limited information about their function has been obtained. In many cases, the ORFs have been predicted from complete genomes using gene-finding programmes and the genomic sequence of hORFs does usually not comprise any homology to other genes. These ORFs are transcribed, but are non-coding (Kowalczuk et al., 1999). The number of hORFs almost doubled in presence of cordycepin.

Recently, cryptic unstable transcripts (CUTs) have been characterised as short (300-600 nt) noncoding RNA transcripts that correspond to inter- and intragenic regions of the genome (Wyers et al., 2005). 10% of intergenic transcripts in *S. cerevisiae* may represent CUTs. They are highly unstable and therefore hardly detectable in wild-type cells. CUTs can be visualised by disrupting *TRF4*, the poly(A) polymerase subunit of the TRAMP complex or *RRP6,* the nonessential subunit of the nuclear exosome. To analyse whether cordycepin might have an effect on the stabilisation of CUTs, Northern blot analysis was carried out. In wild-type cells, the CUT *NEL025c* was not detectable (figure 22). In the two gene deletion strains *ΔTRF4* and *ΔRRP6*, the CUT was detected as a smeary band. It was reported that the RNA transcript of *NEL025c* extends from a defined 5' end to multiple and closely spaced 3' end which are polyadenylated by Trf4p or Trf5p (Wyers et al., 2005). The destabilising effect of polyadenylation can be carried out by Trf4p or Trf5p, explaining the stronger signal of *NEL025c* in the *ΔRRP6* mutant. In presence of cordycepin, the *NEL025c* transcripts were extended and a signal of significant longer transcripts was observed. These extended transcripts may arise from the same two possible mechanisms as proposed for the extended transcripts observed in *ACT1*, *ASC1* and *CYH2* mRNA: Cordycepin could either interfere with poly(A) site selection with the tendency to

use the more distal poly(A) sites more often or cordycepin could have a stabilising effect on the elongated transcripts.

1.7.2 The effect of cordycepin in the *pap1-1* mutant strain

In this microarray analysis, RNA extracted from wild-type cells was directly compared with the temperature-sensitive mutant *pap1-1* cultured at permissive temperature. This experiment was carried out to compare the effect of the mutant form of Pap1p with the effect of cordycepin in wild-type cells. Since cordycepin is thought to act as a polyadenylation inhibitor, both conditions are thought to affect the same process and might show a similar gene expression pattern. Since *PAP1* mutants showed resistance to cordycepin the microarray data might give insight into the cellular effect of the *pap1-1* mutation. The analysis of the microarray data revealed that 323 RNAs were increased and

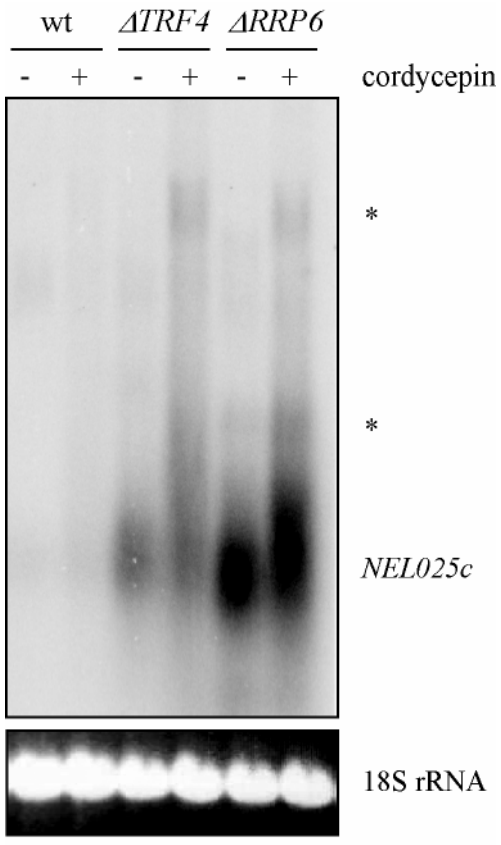

Figure 22 Cordycepin stabilises the CUT *NEL025c*.

Northern blot of wild-type, ΔTRF4 and ΔRRP6 strains which were shifted to 20 μg/ml cordycepin for 60 minutes. Total RNA was extracted and a Northern blot was performed. The membrane was hybridised with random-prime labelled probes against the CUT NEL025c. The asterisks mark the elongated CUT transcripts. 18S rRNA served as control.

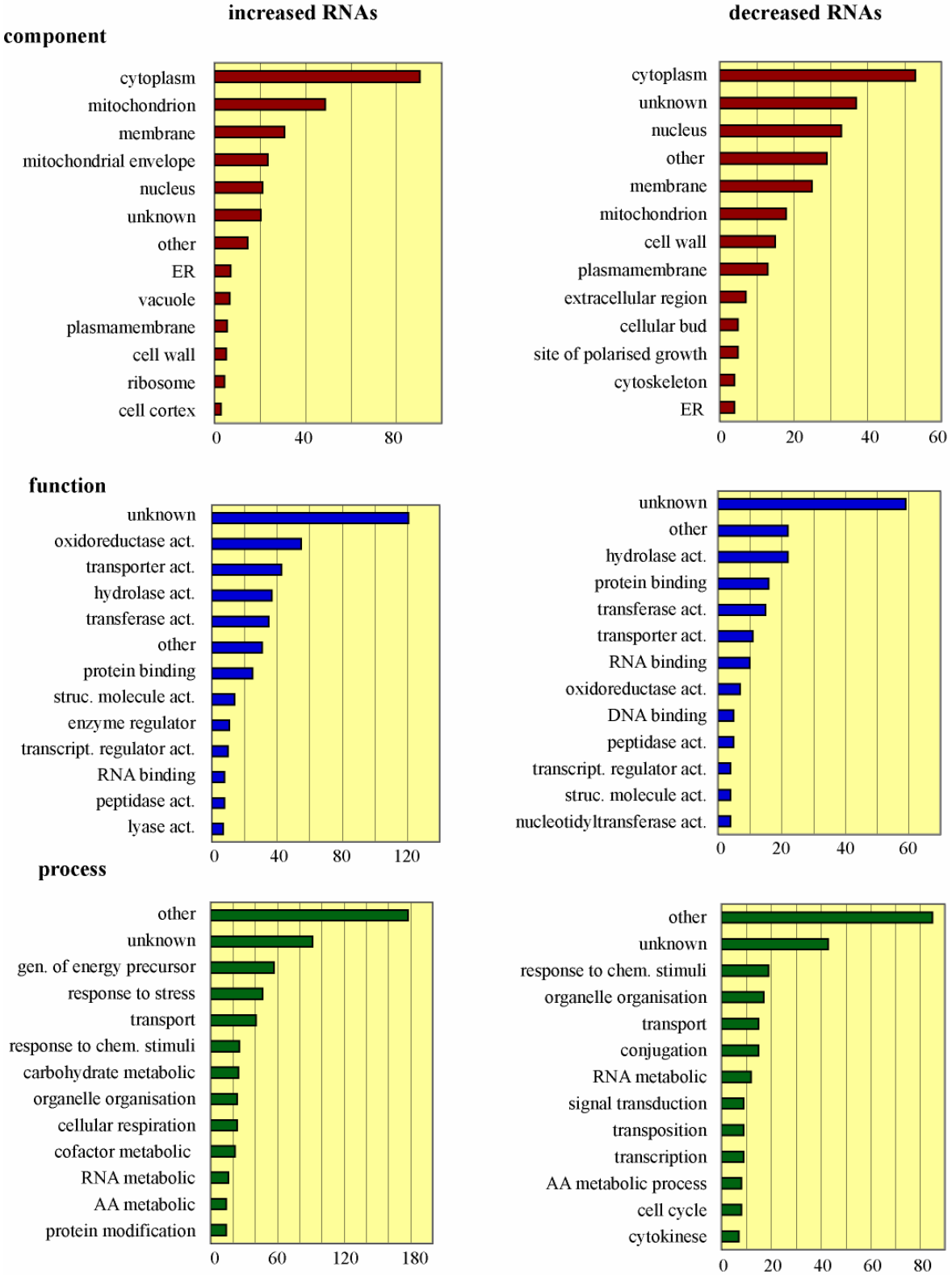

Figure 23 *Pap1-1* mutation seems to enrich RNA transcripts assigned to the mitochondrion.

Increased and decreased RNA in wild-type strains after cordycepin addition were analysed according to the annotated compartment, function and process of the respective protein. The numbers reflect the amount of RNAs annotated to the specific term.

144 RNAs were decreased more than two-fold in the *pap1-1* mutant compared to the wild-type S228C.

The RNAs were grouped according to their compartment and function (figure 23) and interestingly, 122 of the 323 increased RNAs were assigned to the mitochondrion. And even more interestingly, RNAs involved in the process of ATP synthesis and oxidative phosphorylation were highly enriched in the increased RNA fraction (table 4).

	Gene ontology term	**p-value wt/*pap1-1***	**p-value wt/*pap1-1* + Co**
component	mitochondrial membrane part	9.16 E -28	1.75 E -23
	mitochondrial respiratory chain	1.73 E -26	2.45 E -21
	mitochondrion	3.69 E -22	2.13 E -16
	proton-transporting ATP synthase complex	8.97 E -15	7.52 E -16
	proton-transporting two-sector ATPase complex	1.01 E -09	3.14 E -10
function	hydrogen ion transmembrane transporter activity	1.84 E -24	3.04 E -20
	monovalent cation transmembrane transporter	5.75 E -23	6.55 E -19
	oxidoreductase activity	6.21 E -23	2.79 E -20
	cation transmembrane transporter activity	1.07 E -14	1.61 E -11
	ion transmembrane transporter activity	1.08 E -14	9.78 E -11
	hydrogen ion transporting ATP synthase activity	2.39 E -10	8.98 E -10
process	oxidative phosphorylation	1.43 E -40	1.19 E -38
	generation of precursor metabolites and energy	2.69 E -35	1.42 E -33
	electron transport chain	3.17 E -25	2.99 E -20
	respiratory electron transport chain	3.17 E -25	2.99 E -20
	ATP synthesis coupled electron transport	3.17 E -25	2.99 E -20
	oxidation reduction	3.17 E -25	2.99 E -20
	phosphorylation	4.22 E -19	3.25 E -17
	ATP biosynthetic process	2.52 E -14	2.13 E -15
	purine nucleoside triphosphate biosynthetic	2.55 E -13	2.99 E -14
	proton transport	1.80 E -12	2.68 E -13
	hydrogen transport	1.80 E -12	2.68 E -13
	ribonucleoside triphosphate biosynthetic process	1.80 E -12	2.68 E -13

Table 4 *PAP1* mutation leads to an increase of RNAs involved in ATP synthesis.

Enriched gene ontology terms of increased RNAs in pap1-1 cultured at permissive temperature with the respective p-value.

This observation may indicate that a less effective Pap1p reflects a low ATP status and might therefore stimulate the cell to increase of ATP synthesis.

If *pap1-1* mutants were cordycepin resistant because of elevated ATP synthesis, the addition of ATP to the media might suppress the resistance. However, yeast does not have an adenosine transporter (Mäser et al., 1999). Therefore, *pap1-1* and *pap1-2* mutants were dropped on plates containing cordycepin plus additional adenine. Elevated adenine could suppress the cordycepin sensitivity in the wild-type strain (Figure 24). But no effect on the resistant growth of the *PAP1* mutants was observed, independently of the temperature at which they were incubated (except that *pap1-1* didn't grow at 33 °C). In summary, the effect of cordycepin on growth could be diminished by the addition of adenine in wild-type cells, but had no effect on *PAP1* mutants.

RNA levels of *pap1-1* cells shifted to cordycepin for 60 minutes were directly compared to wild-type RNA levels. This gene expression pattern could then be compared to the data from *pap1-1* mutants, gaining further insight into the effect of cordycepin. The microarray analysis revealed 361 increased and 126 decreased RNAs relative to the wild-type. These increased and decreased RNAs were found to be highly similar to those observed in the *pap1-1* cell without cordycepin (table 4). Therefore, cordycepin has no effect in this mutant strain.

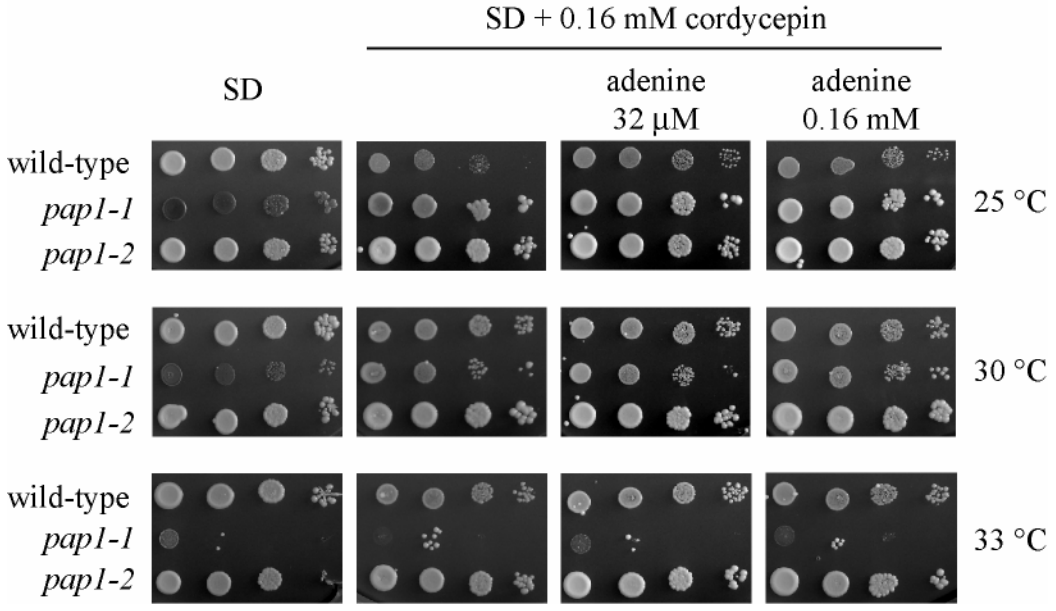

Figure 24 Supplementation of adenine suppresses cordycepin sensitivity of wild-type cells.

Ten-fold dilutions of yeast strains as indicated on the left were dropped on SD plates containing the indicated supplements and incubated at the displayed temperature for 3 days.

1.7.3 Effect of *PAP1* shutoff

In this microarray analysis, RNA extracted from wild-type cells was compared with the temperature-sensitive mutant *pap1-1* shifted to restrictive temperature for 60 minutes. The analysis of the microarray data revealed that 580 RNAs were increased and 870 RNAs were decreased more than two-fold. In the decreased RNA fraction, the component of the ribosome and the process of translation was highly enriched (table 5). No other process or function was significantly enriched in the decreased or increased RNA pool. However, the shutoff with *pap1-1* requires a temperature shift, which has been shown to cause even in a wild-type strain a rapid decrease in mRNA levels (Herruer et al., 1988; Li et al., 1999). This decrease appears to be due to a rapid and transient arrest of general transcription in yeast cells resulting in an overall decrease in mRNA levels (Warner, 1999). Levels of ribosomal protein transcripts are affected most prominently (Wang et al., 2002). In addition to having short inherent half-lives, these transcripts comprise roughly 50% of the mRNA of actively growing yeast (Li et al., 1999). In summary, the data of this array must be compared to data obtained from a wild-type strain shifted to 37 °C, otherwise the effect of the transcriptional decay of ribosomal RNA might be too prominent to detect any other change in RNA levels.

	gene ontology term	**p-value**
compartment	cytosolic ribosome	2.03 E -84
	ribosomal subunit	2.66 E -56
	ribosome	3.06 E -52
	large ribosomal subunit	1.29 E -46
	small ribosomal subunit	9.16 E -35
	ribonucleoprotein complex	1.07 E -26
function	structural constituent of ribosome	3.53 E -58
	structural molecule activity	8.67 E -41
process	cellular biosynthetic process	4.98 E -25
	translation	2.12 E -16

Table 5 Shutoff of *PAP1* leads to a decrease of RNAs encoding ribosomal compounds.

Enriched gene ontology terms of decreased RNA in pap1-1 after 60 minutes at restrictive temperature with the respective p-value.

2 Systematic and genome-wide screening for factors involved in transcription and associated pre-mRNA processing with the adenosine analogue cordycepin

Although the core machinery for pre-mRNA 3' end formation and transcriptional termination has been well characterised (Proudfoot, 2004), it seems likely that additional proteins may act at the interface between transcription and processing. Gene deletions that render cells hypersensitive or resistant to a specific drug can lead to the identification of pathways that buffer the cell against the toxic effects of the drug and might thereby also provide clues about its mode of action (Lum et al., 2004; Parsons et al., 2004). To identify such proteins the yeast knock out collection was screened for growth phenotypes in the presence of the RNA chain terminator cordycepin. The yeast gene deletion library consists of 4825 strains which contain each a deletion in a non-essential gene. Two different screening approaches were carried out: a simple drop test and a functional profiling screen.

2.1 Drop test screen with cordycepin

In the simple drop test, a small liquid culture of each gene deletion strain was dropped on synthetic complete plates containing or lacking cordycepin. Growth of individual strains was analysed after incubation at 30 °C for three days (figure 25). All strains from the gene deletion library were initially screened by a single drop test with a cordycepin concentration of 20 µg/ml. The identified strains were then re-examined on plates containing 20 µg/ml or 40 µg/ml with a drop test were three ten-fold dilutions of the initial culture were used. With the drop test, 155 cordycepin sensitive strains and 9 resistant strains were identified. Resistant growth on a plate is very difficult to identify by eye, which might be the reason for the low number of identified resistant strains (table 6).

2.2 Functional profiling

The second approach was a chemical profiling screen. The yeast gene deletion library was constructed by the homologous recombination of each non-essential gene locus with an individual *kanMX* deletion cassette conferring G418 resistance (figure 26) (Giaever et al., 2002). The *kanMX* marker contains two distinct 20 nucleotide sequences (UPTAG and DNTAG) that serve as molecular bar codes to uniquely identify each mutant. These individual tags are flanked by two 17-19 nucleotide sequences (blue), which are identical for all constructed gene deletion strains. This allows the pooling of all gene deletion strains and their simultaneous analysis. The pool of mutants can be subjected to selection, for example in the presence of cordycepin. Mutants that are sensitive to cordycepin will grow slower and will be underrepresented in the culture applied to selection compared to the non-treated control. On the other hand, mutants which are resistant to cordycepin will grow faster and will be overrepresented in the cordycepin containing culture compared to the reference. After isolation of genomic DNA, the individual bar codes from each culture can be amplified by PCR and labelled by using differently tagged nucleotides. The labelled probes are pooled and hybridised on an oligonucleotide array. The growth of each strain in presence of selective pressure can thereby be directly compared to its reference growth.

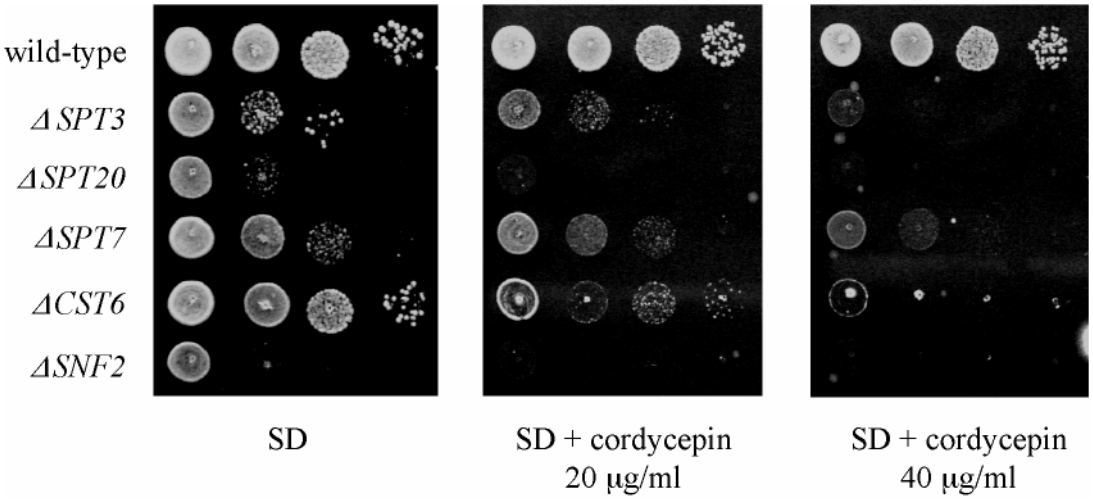

Figure 25 Cordycepin responsive strains.

Ten-fold serial dilutions of the yeast strain indicated on the left were dropped on SD plates containing the indicated cordycepin concentrations. The plates were incubated at 30 °C for 3 days.

The big advantage of this screening approach is that the fitness of each gene deletion strain can be quantitatively scored and already small changes in growth can be detected. However, gene deletions, which cause a strongly reduced growth will be strongly diluted in the culture and no signal can be obtained from these strains (around 300 – 400 strains).

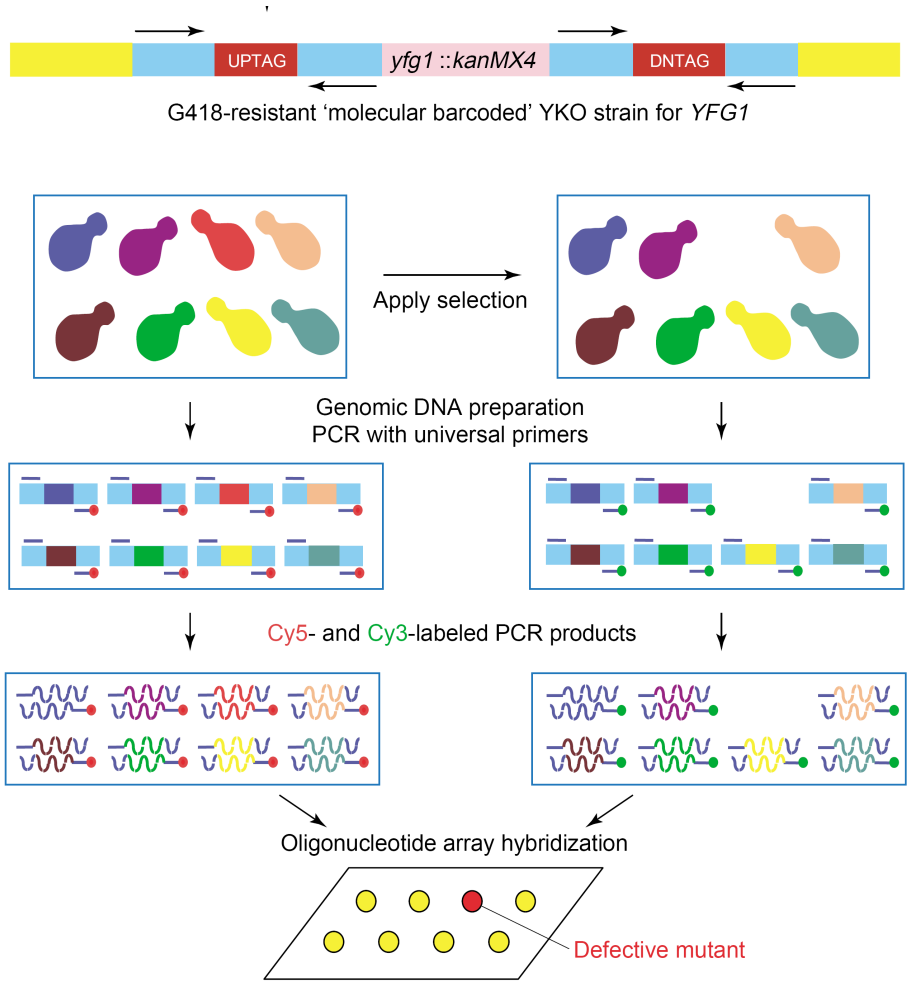

Figure 26 Principle of the functional profiling (Ooi et al., 2006).

A) The kanMX deletion cassette (not in scale) integrated in each gene deletion strain. It contains the kanMX marker (pink) and two unique barcodes (red; UPTAG and DNTAG), which are flanked by two universal priming sites (blue). The genomic sequence surrounding the integrated cassette is shown in yellow. ***B)*** *Schematic outline of the functional profiling assay. Gene deletion strains are pooled and analysed in parallel. After a competitive growth, genomic DNA is extracted and the unique barcodes are amplified using differently labelled universal primers. Hybridisation to an oligo array reveals the relative abundance of each gene deletion strain in the two cultures.*

Optimal results are obtained with this method with an applied selective pressure that causes a growth inhibition of 5 – 10% on wild-type cells (Alain Jaquier, personal communication). To find the respective cordycepin concentration growth analysis was performed. Wild-type strain was subjected to different cordycepin concentrations and growth of the cultures was monitored by measuring the OD_{600} every hour (figure 27). The cultures were kept in exponential growth phase and diluted when reaching an OD_{600} of 0.5. From the resulting growth curves it was deduced that 20 µg/ml cordycepin caused an inhibition of wild-type growth of around 10%.

The yeast gene deletion cell pool used for this experiment was obtained from Alain Jaquier from the Institute Pasteur in Paris. The cultures were grown in duplicates, and yeast strain pools were collected after 12 and 18 generations. The labelling of the probes and the microarray hybridisations were carried out in the lab of Alain Jaquier. The microarray data from the functional profiling were defined as the $\log_2$ value of the ratio +Cordycepin/reference. All gene deletion strains with a value of 1 or higher were considered cordycepin resistant.

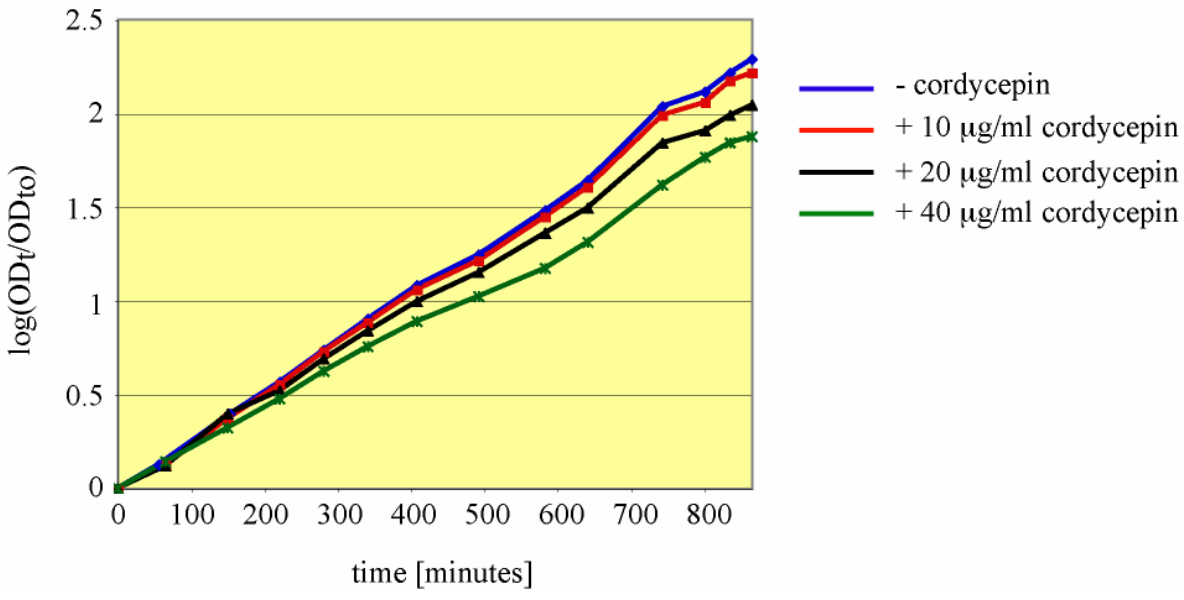

Figure 27 Growth curve of wild-type yeast subjected to different cordycepin concentrations.

The graph represents the growth behaviour of wild-type strains at 30 °C subjected to different cordycepin concentrations. The strains were grown in exponential growth and diluted if they reached an OD of 0.5. The OD was measured every hour and the growth determined as the $\log(OD_t/OD_{to})$.

The gene deletion strains with a value of -1 or smaller were defined as cordycepin sensitive. After 12 generations, 132 strains were identified as sensitive to cordycepin and 73 as resistant. After 18 generations, 281 strains were sensitive and 215 strains were resistant (table 6). The numbers of identified strains more than doubled from generation 12 to generation 18, indicating that also slight growth changes were identified. However, initially we focused for further analysis mostly on the data generated after 12 generations. Almost all strains that were identified as cordycepin sensitive or resistant after 12 generations appeared as well after 18 generations.

	sensitive	overlap	resistant	overlap
drop test	155	34	9	3
functional profiling 12 generations	132	34	73	3
functional profiling 18 generations	281	34	215	3

Table 6 Identified cordycepin sensitive and resistant gene deletion strains.

Overlap describes the number of gene deletion strains that were identified in the drop test and the function profiling screening approach.

2.3 Analysis of the screening results

Parsons and co-workers presented a functional profiling analysis in *S. cerevisiae* using 82 compounds and natural product extracts (Parsons et al., 2006). Combining all obtained chemical-genetic profiles, they identified a set of 121 genes whose corresponding deletion mutant displayed statistically significant multi drug sensitivity. These multi drug sensitive genes include genes whose deletion leads to an increased membrane permeability, for example *ERG5* encoding a drug-efflux pump or *PDR1*, which encodes for a transcription factor that regulates gene involved in multi drug resistance (Parsons et al., 2006). 173 gene deletion strains were identified as multi drug resistant. Most of these strains have a deletion in a gene encoding for a ribosomal protein and were generally growing slowly, which

apparently resulted in the observed multi drug resistance phenotype. Genes whose deletion provokes a multi drug resistance or sensitivity were removed from the identified cordycepin sensitive of resistant mutants resulting in decreased numbers in the list (table 7).

	sensitive	overlap	resistant	overlap
drop test	143	30	8	3
functional profiling 12 generations	123	30	55	3
functional profiling 18 generations	262	30	182	3

Table 7 Identified cordycepin responsive gene deletion strains after the removal of multi drug sensitive and multi drug resistant strains.

From tables 4 and 5 it is quite obvious that the overlap of the two screen results is rather small. Apparently, the environment of the yeast cells plays a tremendous role for the output of a screen. For the drop test screen, the yeast cells were grown on plates for 3 days, allowing the cells to exit exponential growth phase. In contrast, the yeast cells were grown in a liquid culture and kept in exponential growth phase for the functional profiling screen. Furthermore, the yeast gene deletion strains were pooled and cultured in a competitive manner. These circumstances could influence the result of a screening procedure. Moreover, many strains that were identified in the drop test but not in the functional profiling screen were lost because of the general slow growth of the mutant.

The genes whose deletion causes cordycepin sensitivity or resistance were analysed according to their gene ontology (GO) terms in the category compartment, function and process (figure 28). Cordycepin seems to affect all cellular compartments and molecular processes, as the identified genes were not restricted to the compartment of the nucleus or the process of transcription. One could argue such a central process as transcription and mRNA formation might provoke global effects. The relative abundance of the respective GO terms was very similar for the genes resulting from the drop test and for those from the functional profiling. The additional genes identified in the functional profiling after 18 generations were not uniformly distributed with regard to the GO term in the case for

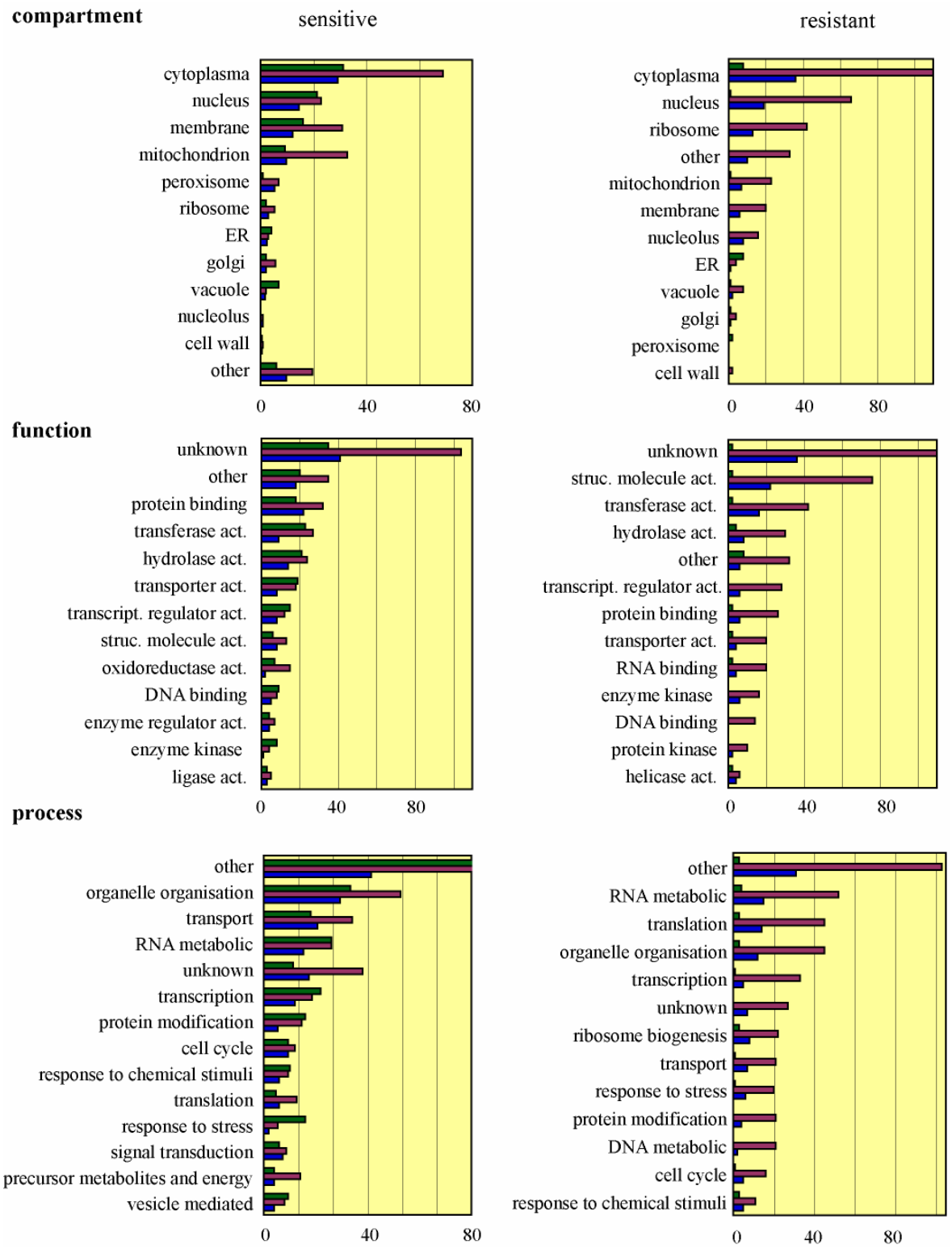

Figure 28 Cordycepin sensitive and resistant gene deletion strains grouped by gene ontology terms.

The most abundant gene ontology terms for component, function and process are shown.

cordycepin sensitive gene deletions. Genes localised to the cytoplasm, membrane and mitochondria having an unknown function were significantly enriched. For the genes whose deletion causes cordycepin resistance, these additional genes were distributed more uniformly.

Some significantly enriched GO terms associated with cordycepin sensitivity are symbolised by a few examples in table 8. In both screens, genes that belong to a chromatin remodeler and which are involved in the process of the chromosomal architecture were highly enriched, especially subunits of the *SWR1* complex. This complex mediates the ATP-dependent exchange of histone H2A for the H2A variant *HZT1* leading to transcriptional regulation of selected genes by chromatin remodelling (Mizugushi et al., 2004). Interestingly, the gene deletion strain *(ΔHTZ1*) lacking this histone variant was as well identified as cordycepin sensitive.

The term 'RNA biosynthetic process' is enriched in the sensitive part of the drop test only, and these genes function in different stages of transcription: *SPT3*, a subunit of the transcriptional activator complex SAGA, *SPT4*, a transcriptional elongation factor, the *SWD1* subunit of the COMPASS complex, and *RAI1*, interacting with Rat1p and involved in transcription termination. This result may indicate that a screen using the adenosine analogue cordycepin can identify factors involved in transcription and associated pre-mRNA processing.

A third group of genes, which appeared only in the functional profiling screen, could be localised to peroxisomes and are involved in peroxisome biogenesis or transport into peroxisomes. The peroxisomes are small organelles participating in the metabolism of fatty acids and most interestingly, also in the catabolism of purines (Wanders and Waterham, 2006). Genes necessary for proxisome biogenesis are termed *PEX* genes (Kiel et al., 2006). Some of these *PEX* genes deletion strains displayed a strong sensitivity towards cordycepin. It might be that CoTP is also degraded in the peroxisomes. However, when genes important for peroxisome integrity and biogenesis were deleted, the process of purine catabolism could be carried out less efficiently. This might also lead to a less efficient CoTP degradation and a subsequent higher CoTP concentration in the cell. To test whether strains lacking *PEX* genes were also cordycepin sensitive in the drop test, some strains lacking a *PEX* gene were dropped on plates lacking or containing cordycepin (figure 29). The tested strains all exhibited reduced growth in presence of cordycepin, indicating that peroxisomes might be involved in the suppression of cordycepin toxicity.

Component	GO term	p-value	genes
drop test	chromatin remodeling complex	2.42E-10	*SWC3 SWD1 HPC2 SWC5 SWR1 RSC1 SNF6 HIR3 BRE2 VPS71 ARP5 YAF9 HTZ1 HIR2 ARP8 SNF2*
	vacuolar proton-transporting ATPase complex	1.89E-07	*VMA2 TFP1 VMA8 PPA1 VMA6 VMA4 TFP3 VMA13*
FP 12	peroxisome	8.09E-08	*PEX22 PEX19 PEX5 PEX10 PEX3 PEX14 PEX4 PEX2 PEX1 PEX13 PEX12*
	chromatin remodeling complex	1.03E-06	*SWC3 LDB7 HPC2 SWC5 SWR1 VPS72 HIR3 BRE2 ARP6 VPS71 HTZ1 HIR2*
	SWR1 complex	8.90E-07	*SWC3 SWC5 SWR1 VPS72 ARP6 VPS71*
FP 18	mitochondrial inner membrane	1.51E-13	*COR1 CBS1 CBS2 COQ4 COX20 BCS1 SHE9 QCR7 RIP1 PET117 PET122 QCR6 COX18 SHY1 PET54 COX6 MDM31 YIA6 PET191 ATP7 PAM17 COX1*
	peroxisome	3.72E-07	*PEX22 PEX19 PEX5 PEX10 PEX3 PEX14 PEX8 PEX4 PEX2 PEX1 PEX13 PEX12 MLS1 PEX17 PEX6 PEX15 PEX25*
	mitochondrion	3.32E-06	*SWC3 CYC3 GCV3 COR1 COQ1 RTC2 PDB1 YBR238C MRPL37 MRPL27 YCP4 IMG2 CBS1 CBS2 COQ4 COX20 MRP1 BCS1 SHE9 QCR7 RIP1 CIN8*
	SWR1 complex	1.85E-06	*SWC3 SWC5 SWR1 VPS72 ARP6 VPS71 YAF9*
Process			
drop test	maintenance of chromatin architecture	1.97E-11	*SWC3 SWD1 SPT7 HPC2 SWC5 BRE1 UME6 SUM1 SWR1 SPT3 ADA2 RAD6 RSC1 SPT4 SNF6 HIR3 BRE2 VPS71 ARP5 YAF9 HTZ1 HST1 SPT20 HIR2 ARP8 NPT1 RFM1 SNF2 LGE1*
	RNA biosynthetic process	4.93E-07	*SWD1 HPC2 BRE1 REG1 REF2 UME6 SUM1 SPT3 RPB9 SNF4 RAI1 RSC1 SPT4 GTR2 BUD32 SNF6 STB5 CST6 GON7 HIR3 BRE2 SPT8 YAF9 SIN4 PHO80 HTZ1 HST1 THP1 HIR2 NPT1 RFM1 SNF2 PHO85*
FP 12	peroxisomal transport	2.05E-14	*PEX22 PEX19 PEX5 PEX10 PEX3 PEX14 PEX4 PEX2 PEX1 PEX13 PEX12 PEX6 PEX15*
	histone exchange	5.86E-06	*SWC3 SWC5 SWR1 VPS72 ARP6 VPS71*
FP 18	peroxisomal transport	9.01E-17	*PEX22 PEX19 PEX5 PEX10 PEX3 PEX14 PEX8 PEX4 DJP1 PEX2 PEX1 PEX13 PEX12 PEX17 PEX6 PEX15 PEX25*
	histone exchange	2.04E-05	*SWC3 SWC5 SWR1 VPS72 ARP6 VPS71 YAF9*
	mitochondrial respiratory chain complex assembly	2.74E-06	*COX20 QCR7 PET117 SHY1 CBP4 PET191 COX17 COX19 COX12*

Table 8 Significantly enriched GO terms with the respective p-value of the identified genes whose deletion causes cordycepin sensitivity.

After 18 generations, genes localising to the mitochondria involved in cellular respiration and in the respiratory chain complex IV assembly were also identified as significantly enriched. The sensitivity caused by the deletion of these genes may only slightly hinder growth, since they were not enriched after 12 generations. This is very interesting, since mitochondria are the main sites of cellular ATP synthesis. This may imply that these strains have less a higher CoTP to ATP ratio possibly increasing the chance for an enzyme to choose CoTP instead of ATP as a reaction partner. This could lead to the observed cordycepin sensitivity of these strains. Additionally the drop test showed significantly enriched genes of the vacuolar ATPase complex and vacuolar proteins involved in the pH regulation. The lack of these vacuolar genes could also have an impact on relative ATP levels or relative ATP abundance, which might be the cause for the observed cordycepin sensitivity.

Since both screening approaches were carried out to identify new factors involved in 3' end formation the results from both screenings were combined and further experiments were focussed on genes localising to the nucleus or having an unknown function. Figure 30 summarises the selected genes and their function. In this selection, the consensus of the two screening results is also relatively small and. However, subunits of the chromatin remodeler SWR1 or SWI/SNF complex, genes involved in chromatin modification (SAGA complex, SET1 complex), general transcription factors and repressors, and genes having a function in mRNA processing and export can be found in

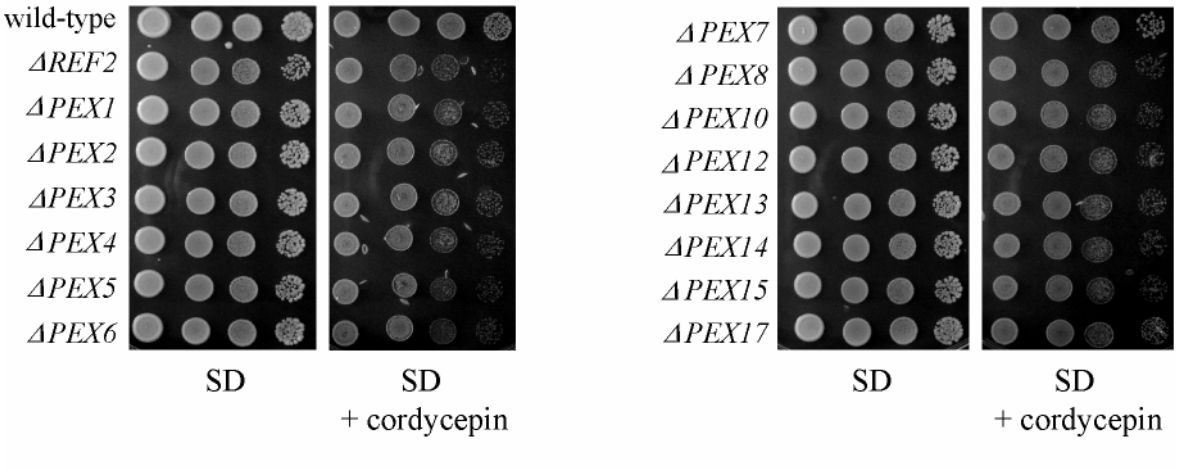

Figure 29 *PEX* mutants are cordycepin sensitive.

Ten-fold serial dilutions of the yeast strain indicated on the left were dropped on SD plates containing 40 µg/ml cordycepin. The plates were incubated at 30 °C for 3 days.

gene	function
AOR1	SWR complex
ARP5	
ARP6	
ARP8	
SWC1	
SWC3	
SWC5	
SWR1	
VPS71	
VPS72	
YAF9	
SNF2	SWI/SNF complex
SNF6	
RSC1	RSC complex
LDB7	
IES6	INO80 complex
HST1	histone deacetylase
HTZ1	histone variant HA2Z
ADA2	SAGA
SPT20	
SPT3	
SPT7	
SPT8	
BRE2	Set complex
SWD1	
CST6	transcription factor
HPC2	
UME6	
HIR2	repressors
HIR3	
RFM1	
SUM1	
CDC40	mRNA processing
LEA1	
MUD2	
PBP1	
RAI1	
REF2	
SPT4	
PAN2	
PAN3	

	drop test
	both
	FP

gene	function
THP1	mRNA export
SAC3	
APQ12	
MOD5	tRNA modifcation
RPB9	transcription
SIN4	mediator complex
BUB1	cell cycle
SIT4	
SMI1	
BUB3	
MAD1	
BIK1	
CIN8	
GTR1	nuclear transport
GTR2	
RAD6	ubiquitination
BRE1	
BMH1	signal transduction
DBF2	
PHO80	
PHO85	
PTC1	
REG1	
SAC7	
SAP155	
SNF4	
TPD3	
TUB3	microtubuli
NPT1	NAD biosynthesis
MKS1	AA biosynthesis
TRP4	
URA4	nucleotide synthesis
DOA1	protein degradation
ISR1	

gene	function
CLG1	unknown
DLT1	
FYV10	
ICT1	
LGE1	
MUB1	
OPI11	
UBP13	
VPS69	
YDR532	
YIM2	
YBR147c	hypothetical protein
YDL172c	
YDL173w	
YDR008c	
YDR271c	
YDR532c	
YEL045c	
YER156c	
YGL024w	
YGL152c	
YGR259c	
YJL175w	
YJL211c	
YKL053w	
YKL118c	
YKL121w	
YLR278c	
YLR358c	
YLR407w	
YNL171c	
YOL050c	
YOL138c	
YOR072w	
YOR331c	
YPR045c	
YPR123c	

Figure 30 Selected genes, whose deletion causes cordycepin sensitivity, sorted according their function.

The colour reflects the respective screening method, drop test only (yellow), functional profiling only (green) and both (red).

this selection. Also the term cell cycle and signal transduction appears and some genes with an unknown function. Moreover, it is unclear whether all hypothetical genes encode for proteins.

In the drop test, strains lacking genes involved in RNA turnover were identified as cordycepin resistant (figure 31). At concentrations up to 120 µg/ml cordycepin no growth phenotype was observed in these strains, although wild-type cells growth was strongly reduced. Pop2p mediates 3' to 5' mRNA deadenylation, Ski3p forms a complex with the putative RNA helicase Ski2p and this complex plays a central role in the cytoplasmic degradation of mRNA mediated by the exosome (Wang et al., 2005). *XRN1* encodes for a cellular 5' to 3' exonuclease that degrades the mRNAs after deadenylation and decapping. Loss of these genes leads to a stabilisation of mRNA and this apparently can suppress cordycepin toxicity. Ado1p is the adenosine kinase in yeast which converts adenosine to AMP. This gene is not essential in yeast, since AMP can still be produced form de novo purine biosynthesis. But cordycepin probably can not be converted into CoTP anymore, and therefore it can not cause termination of RNA synthesis.

In the results from the functional profiling, the term ribosome and translation is enriched in the list of those genes, whose deletion causes cordycepin resistance. Strains lacking a gene involved in ribosomal stability or integrity are cordycepin resistant.

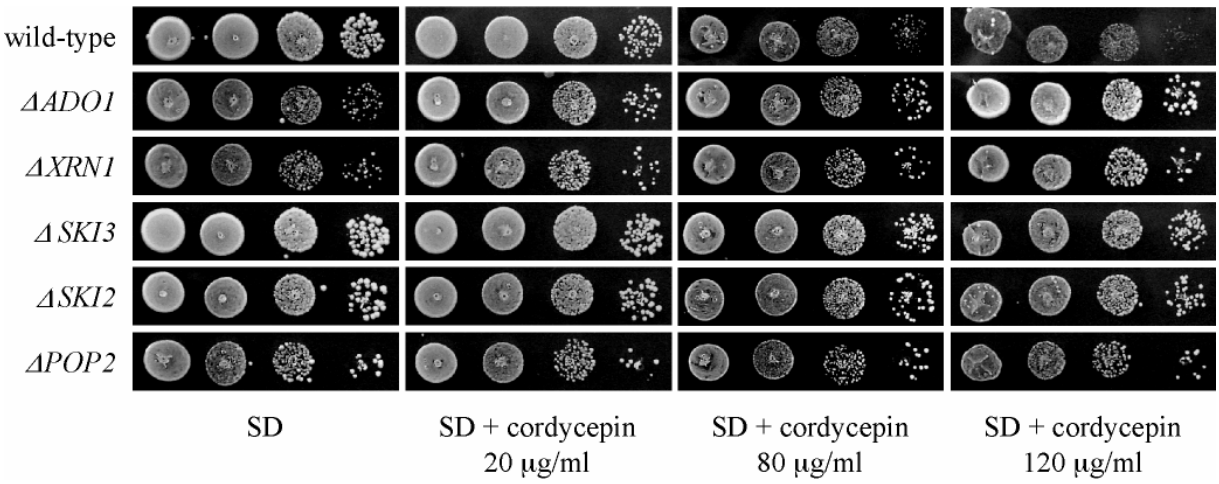

Figure 31 mRNA stabilisation suppresses cordycepin toxicity.

Ten-fold serial dilutions of the yeast strain indicated on the left were dropped on SD plates containing the indicated cordycepin concentration. The plates were incubated at 30 °C for 3 days.

However, many multidrug resistance genes encode for ribosomal proteins, therefore it could be that the deletion of ribosomal proteins in general causes a resistance to many conditions or drugs. Therefore, the ribosomal genes were not further analysed.

2.4 Poly(A) tail analysis

To test whether some of the identified genes function in 3' end formation, the poly(A) tail distribution was analysed in most of the cordycepin sensitive mutants (Martin and Keller, 1998). The poly(A) tails in yeast wild-type cells show a homogeneous distribution from approximately 10 to 70 added As.

Many mutant strains revealed the same poly(A) length distribution, but in some mutants poly(A) tails were altered (figure 32), indicating that the deleted gene might have a function in polyadenylation and 3' end processing. For a few mutant strains, the reason for the altered poly(A) tail structure is known, as for *ΔXRN1*. Strains lacking the cytoplasmic mRNA exonuclease accumulate mRNA with a truncated poly(A) tail resulting from deadenylation as the first step in mRNA turn over (Muhlrad et al., 1995). For most mutant strains with altered poly(A) distribution, the reason for it is not clear. In *ΔTHP1* and *ΔSAC3* strains an accumulation of short poly(A) and additionally extended poly(A) tails were observed. Yeast lacking *THP1* or *SAC3* has been shown to accumulate polyadenylated RNA in the nucleus (Fischer et al., 2002), however, what causes the accumulation of short poly(A) tails is unclear. The most intriguing poly(A) phenotype observed, the lack of any poly(A) tail signal in *ΔPHO80* and *ΔPHO85,* was further analysed and is the principal subject of chapter three.

2.5 Synthetic lethality test

To further concentrate on genes harbouring a possible function in 3' end formation, some selected genes were tested for a possible genetic interaction with *RNA14* in a synthetic lethal test. *RNA14* is an essential gene and is involved in cleavage and polyadenylation (Mandart, 1998). Synthetic lethality describes any combination of two separately non-lethal mutations that leads to inviability (figure 33). A synthetic growth defect indicates a

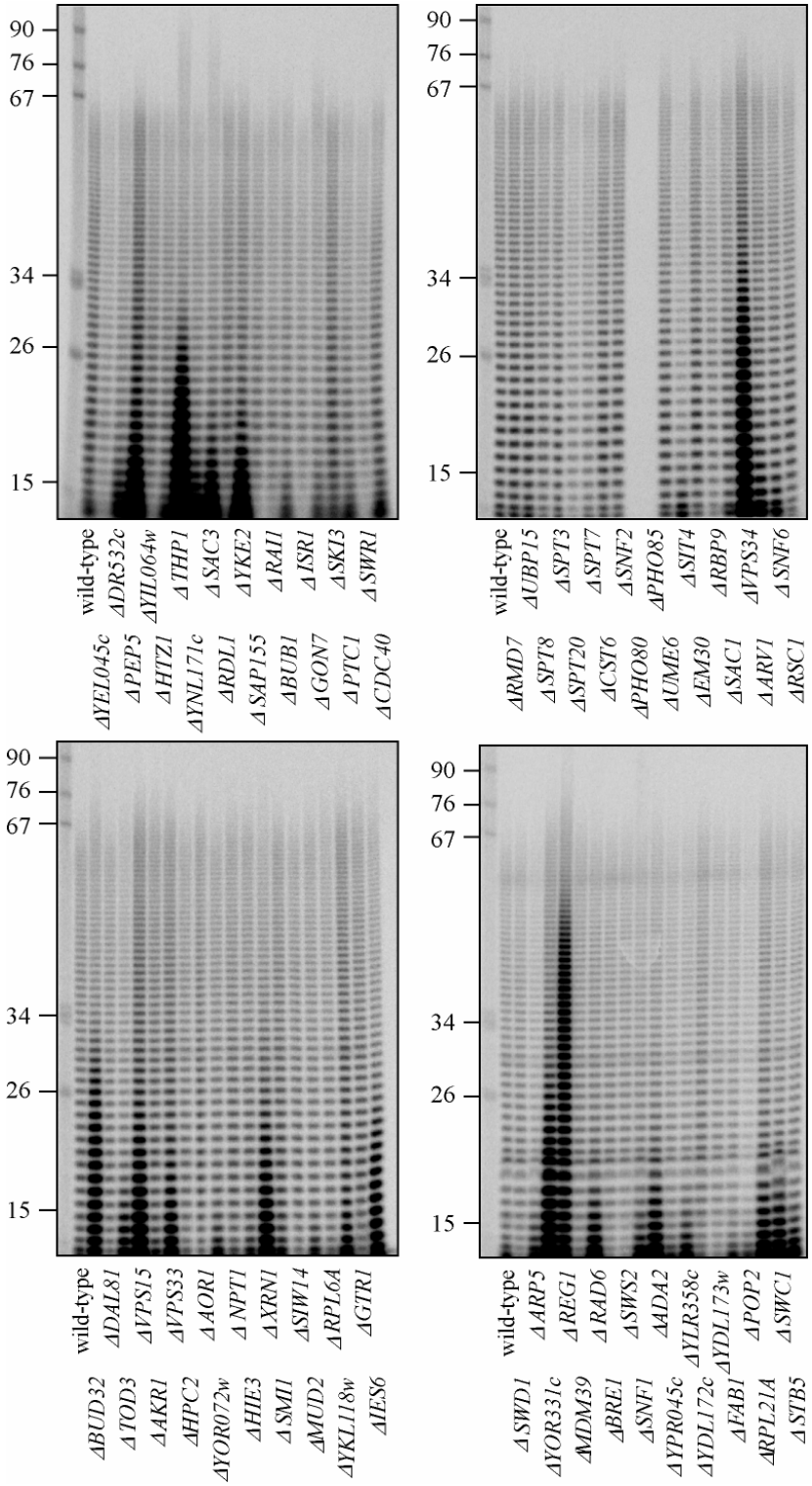

Figure 32 Poly(A) tail length distribution is altered in many cordycepin responsive mutant cells.

Poly(A) tail length distribution assay of cordycepin sensitive mutants after growth at 30°C for 6 hours. Total RNA was extracted and labelled with Pap1p and radioactive cordycepin. The RNA was digested with RNase A and T1 and the remaining poly(A) tails were separated on a 15% polyacrylamid gel. The position and size in number of nucleotides of the marker bands are indicated on the left.

combination of two separate non-lethal mutations and confers a growth defect more severe than that of either single mutation. The interpretation is that a synthetic growth defect reflects an important genetic interaction, whereas synthetic lethality reflects an essential interaction.

In the temperature sensitive mutant *rna14-1* carrying a *URA3* plasmid containing the wild-type *RNA14* gene, genes identified in the screen were deleted. Challenging these cells with 5-flouroorotic acid forces them to loose the plasmid, thus, exhibiting the double mutation. Many of the tested genes were indeed synthetic lethal in combination with *rna14-1* (figure 34). *APQ12* is involved in nucleoplasmatic transport of mRNA and *ΔAPQ12* mutants show a defect in mRNA export (Baker et al., 2004). *THP1* forms a complex with *SAC3,* which showed a growth defect in combination with *rna14-1* and is involved in the coupling of mRNA 3' end formation and export. *HTZ1* is a histone H2A analogue, which is incorporated into chromatin by the SWR1 chromatin remodelling complex, whose *SWR1* subunit exhibited a growth defect in combination with *RNA14*. The *SNF6* and *SNF2* subunits of the SWI/SNF complex exhibited as well a genetic interaction with *RNA14*. The two hypothetical transcripts *YDL172c* and *YDL173w* exhibited both a strong sensitivity against cordycepin and appeared in both screens. These two genes overlap almost completely. *ISR1* encodes for a protein kinase of unknown function but is synthetic lethal with *rna14-1*. *ISR1* displayed a physical overlap with the 3' UTR of *YTH1*, an essential factor of the 3' end machinery. Disrupting *ISR1* might lead to a less efficient *YTH1* mRNA production, which could be responsible for the observed cordycepin sensitivity. Apparently, a gene deletion can also indirectly cause sensitive growth in presence of cordycepin. This example nicely illustrates that using cordycepin for screening the gene deletion library indeed revealed mutants having a defect in 3' end formation.

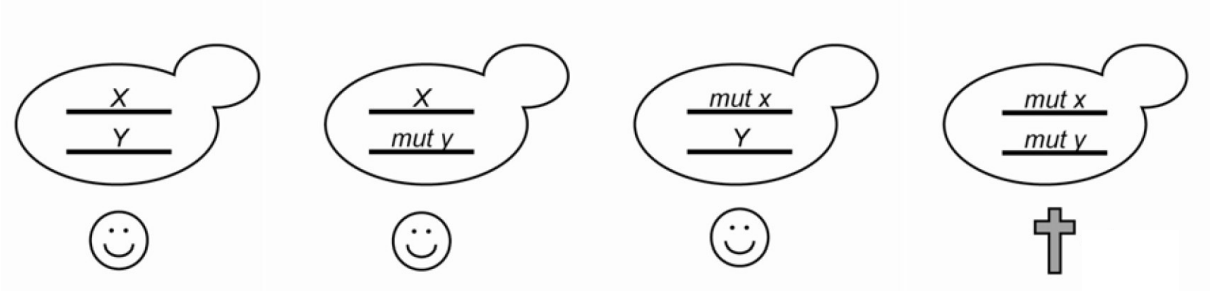

Figure 33 Principle of synthetic lethality.

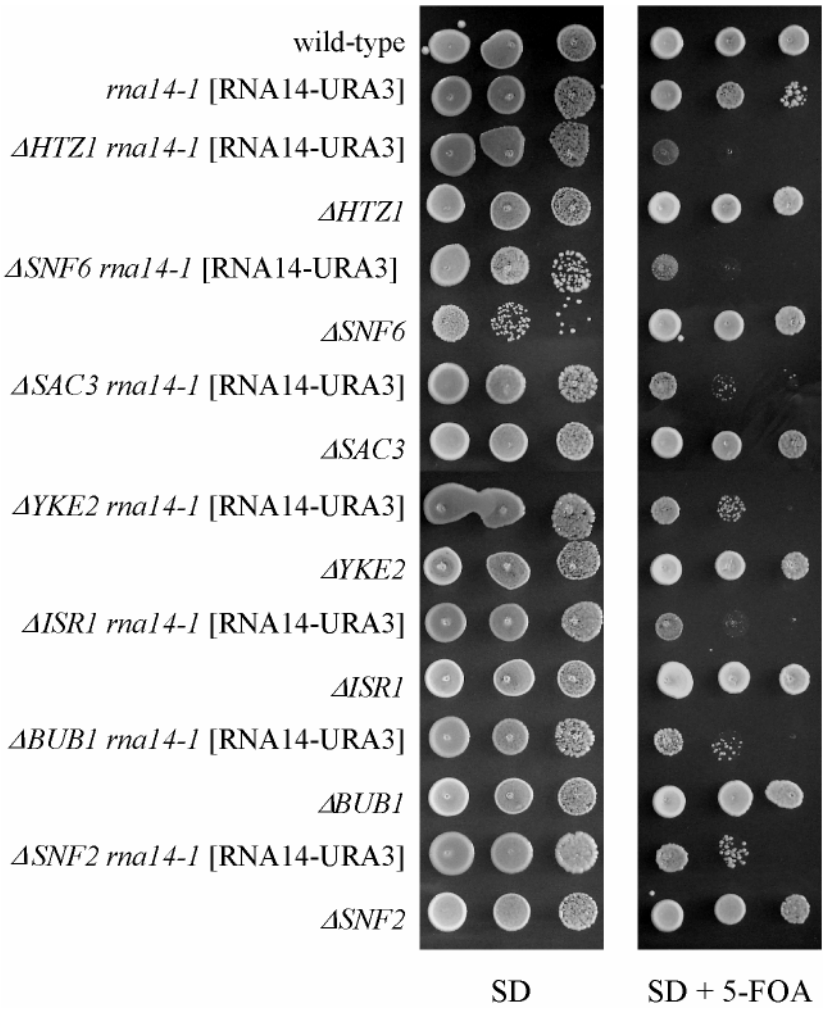

Figure 34 Synthetic lethality test with *rna14-1*.

Ten-fold serial dilutions of the yeast strain indicated on the left were dropped on SD plates containing 1 mg/mg 5' fluoroorotic acid (5-FOA) as indicated. The plates were incubated at 30 °C for 3 days.

effect	**genes**
synthetic lethality	*APQ12, ECM30, HTZ1, ISR1, PHO80, RPB9, SNF6, THP1, RAI1, YDL172c, YDL173w*
growth defect	*AKR1, BUB1, CST6, RMD7, SNF1, SNF2, SPT8, SWR1, SAC3*
normal growth	*GTR1, MUD2, PEP5*

Table 9 Summary of tested genetic interactions with *rna14-1*.

Tested genetic interactions with rna14-1 were listed according to the growth phenotype of the double mutant.

From the tested 23 genes, eleven genes were synthetic lethal, nine genes showed a genetic interaction, but only three genes exhibited no genetic interaction with *RNA14*, suggesting that most of the identified genes might have a synergistic function with respect to *RNA14* (table 9).

2.6 Elongated RNA transcripts in cordycepin sensitive mutants

Several cordycepin sensitive gene deletion strains were selected and tested for the presence of elongated transcripts as had been observed in wild-type cells shifted to cordycepin. Therefore, Northern blot analysis was performed with eleven gene deletion strains before and after the addition of cordycepin to the culture. *CYH2* hybridisation revealed that none of the tested mutant strains exhibited extended *CYH2* transcripts, only upon cordycepin addition longer RNA transcripts were observed (figure 35). Poly(A) site usage was analysed in *ACT1* and *ASC1* mRNA. Ref2p is the only not essential protein of the core

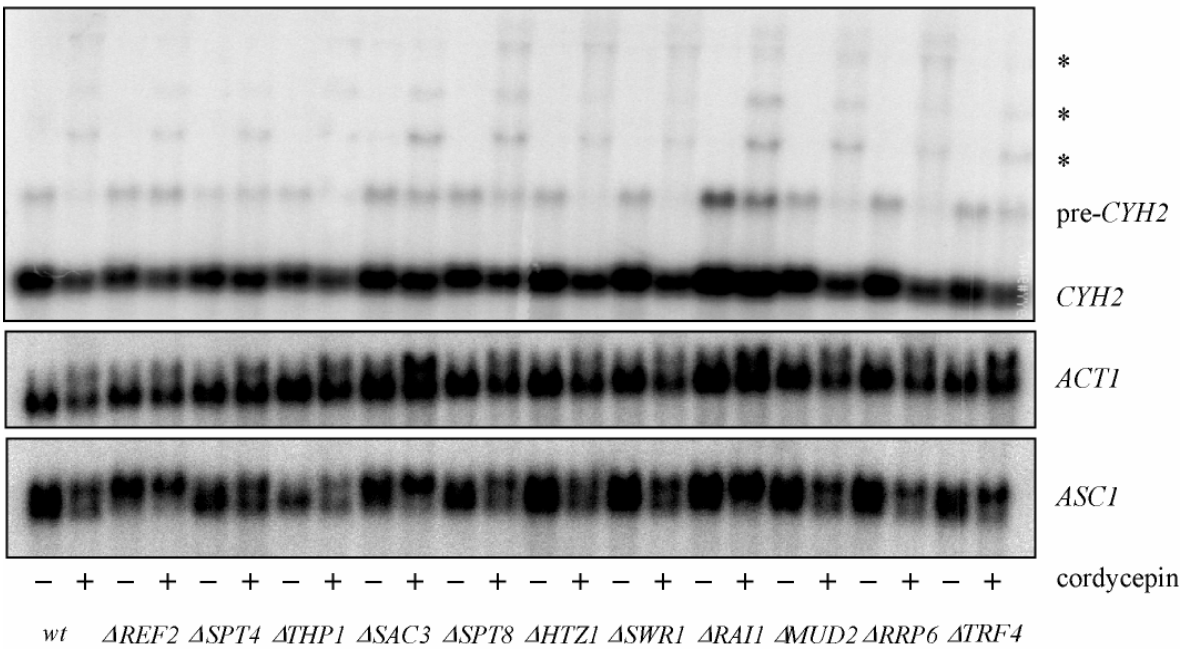

Figure 35 Cordycepin sensitive mutants display an alteration in the poly(A) site usage of *ACT1* and *ASC1* mRNA and elongated *CYH2* transcripts in presence of cordycepin.

Northern blot of total RNA extracted from wild-type and mutant strains cultured in presence of absence of cordycepin [20 µg/ml]. The membrane was hybridized with random-primed labelled probes against ACT1, ASC1 and CYH2 mRNA. The asterisks mark elongated CYH2 transcripts.

3' end machinery and therefore *ΔREF2* mutants showed a preferred usage of more distal poly(A) sites than wild-type cells without the addition of cordycepin. All tested mutants seemed to have a normal poly(A) site usage in the absence of cordycepin, which was altered when cordycepin was added.

Since cordycepin also led to elongated transcripts of the CUT *NEL025c* in the *ΔTRF4* and *ΔRRP6* mutant strains, this transcript was also analysed for the selected mutant strains (figure 36). Although wild-type cells do not accumulate the *NEL025c* transcript in presence of cordycepin, Northern blot analysis revealed that in the *ΔHTZ1* and *ΔSWR1* a smear of a signal was observed in presence of cordycepin, similar to that observed in the *ΔTRF4* mutant. However, further analysis of a possible accumulation of elongated *NEL025c* and possibly also of other CUTs in these two mutant strains is necessary to draw a final conclusion.

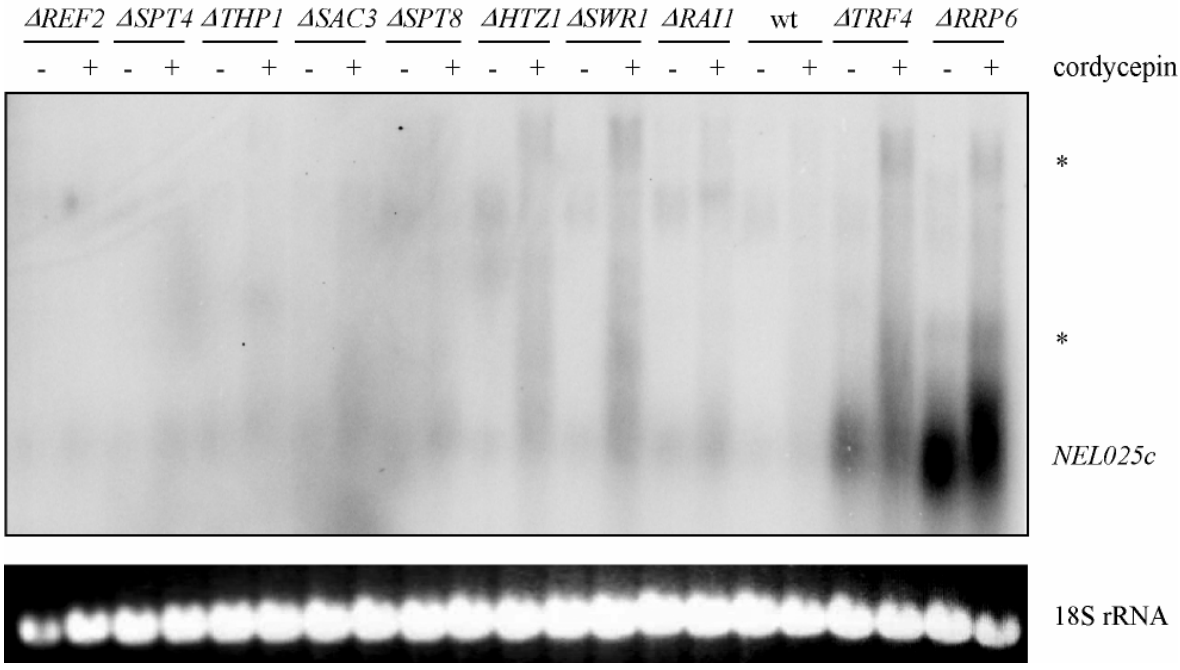

Figure 36 Cordycepin might lead to an accumulation of the CUT *NEL025c* in some cordycepin sensitive mutants

Northern blot of total RNA extracted from wild-type and mutant strains cultured in presence of absence of cordycepin [20 µg/ml]. The membrane was hybridized with random-primed labelled probes against the CUT NEL025c. The asterisks mark elongated NEL025c transcripts. 18S rRNA served as control.

2.7 Poly(A) site shift in cordycepin resistant mutants

Since cordycepin affects poly(A) site usage of *ACT1* and *ASC1* mRNAs in wild-type strains, it was tested, whether cordycepin resistance also implies a suppression of the observed poly(A) site shift. Six cordycepin resistant mutants were cultured and shifted to medium containing cordycepin. *ΔADO1* acted as a control, since cordycepin can not be converted into the active CoTP in this mutant strain and therefore a difference in the poly(A) site usage of *ACT1* should not be detectable. As shown in figure 37, *ΔADO1* displayed no altered poly(A) site usage of the *ACT1* mRNA nor a general decrease in the *ACT1* mRNA level. This indicates, that the conversion to CoTP is necessary to cause a change in poly(A) site usage. The five tested cordycepin resistant mutant strains showed a decrease in the *ACT1* mRNA signal and in addition a shift of the poly(A) site usage in the *ACT1* mRNA. This indicates that cordycepin resistance does not correlate with the observed polyadenylation site shift.

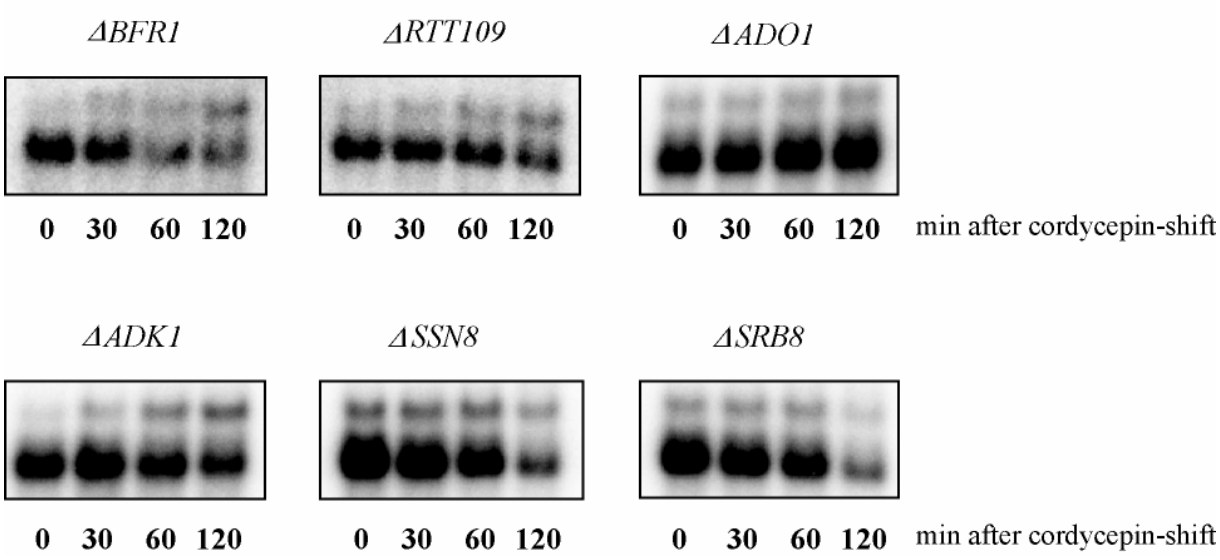

Figure 37 Cordycepin resistance does not correlate with suppression of cordycepin-induced poly(A) site shift.

Northern blot of total RNA extracted from cordycepin resistant mutant strains at the indicated time after the shift to cordycepin [20 µg/ml]. The membrane was hybridized with random-primed labelled probes against ACT1 mRNA.

2.8 Partial suppression of cordycepin toxicity by supplementation of adenine

In the cordycepin resistant *pap1-1* mutant an increased expression of genes involved in ATP synthesis was detected. This suggested that elevated ATP levels might reduce cordycepin toxicity. To test this hypothesis, four selected cordycepin sensitive mutant strains were dropped on plates containing cordycepin additional adenine. *ΔREF2* and *ΔISR1* strains acted as controls as both of these mutants were directly or indirectly associated to 3' end formation. For all four cordycepin sensitive mutant strains, the addition of adenine could only slightly suppress cordycepin toxicity (figure 38), although growth was still clearly reduced compared to growth on minimal media.

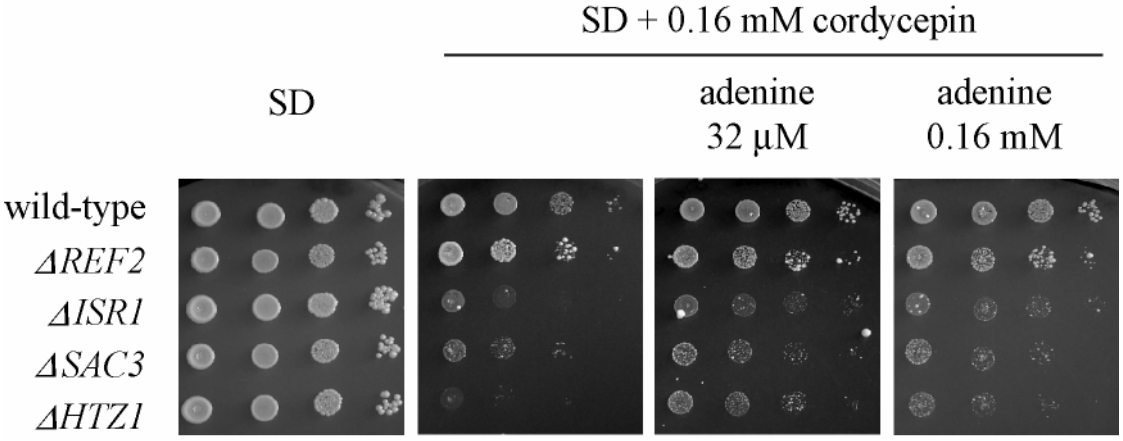

Figure 38 Partial suppression of cordycepin toxicity by the addition of adenine.

Ten-fold dilutions of wild-type and mutant yeast strains were dropped on SD plates containing the indicated supplements and incubated for 3 days.

2.9 *pap1-2* mutation suppresses cordycepin sensitivity

The *pap1-1* mutant strain is resistant to cordycepin, which might be caused by increased ATP synthesis. This opens the question whether a *Pap1* mutation in a cordycepin sensitive gene deletion strain could have an influence on the growth inhibition of these mutants in

presence of cordycepin. Therefore, *APQ12, SAC3* and *THP1* genes were deleted in the *pap1-2* temperature sensitive mutant strain and growth was tested in a drop test. *Pap1-2* doublemutants displayed a restored growth phenotype in presence of cordycepin (figure 39), whereas the three single gene deletion mutants exhibited strongly inhibited growth in presence of the drug. This means that *pap1-2* mutation is able to suppress cordycepin sensitivity in gene deletion strains that are sensitive to cordycepin. Since the *pap1-2* mutation was able to restore growth in presence of cordycepin in these mutant strains in might also suppress the altered cordycepin-induced polyadenylation site usage. Northern blot analysis of *ΔAPQ12 pap1-2* and *ΔSAC3 pap1-2* double mutants revealed that the most proximal polyadenylation site is used almost uniquely (figure 40). Apparently, the *pap1-2* mutation suppresses not only the growth phenotype of these mutant strains, but also restores the cordycepin-induced variation of polyadenylation sites.

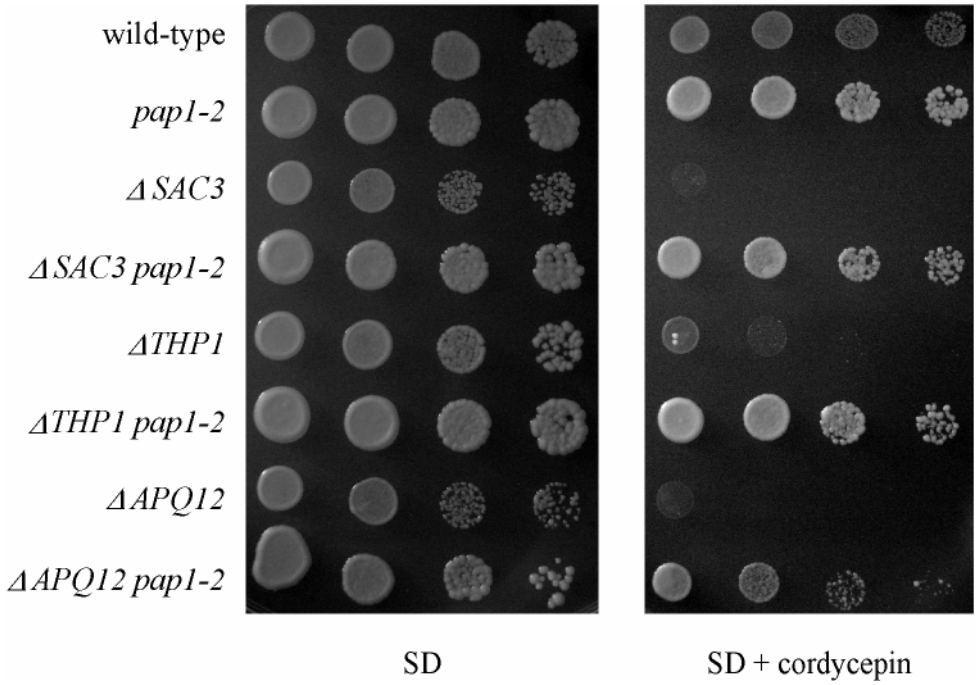

Figure 39 *pap1-2* mutation suppresses growth inhibition in presence of cordycepin in cordycepin sensitive gene deletion strains.

Ten-fold dilutions of single mutants and pap1-2 double mutants were dropped on SD plates with or without 40 µg/ml cordycepin and incubated for 3 days at 30 °C.

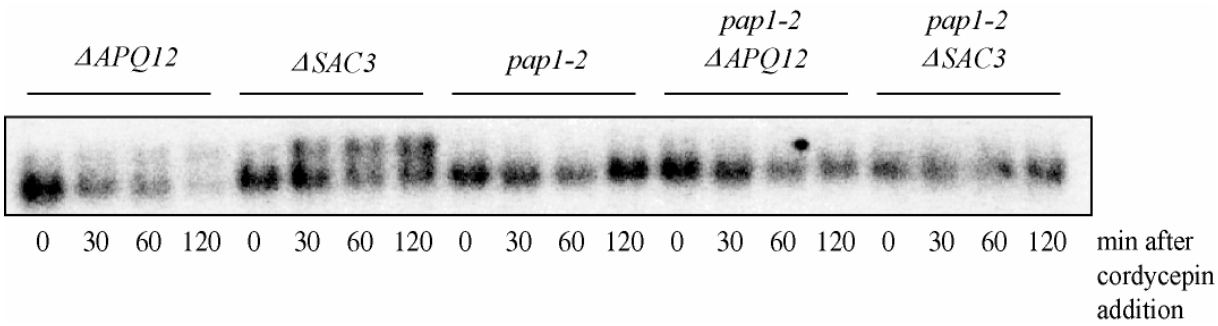

Figure 40 Cordycepin-induced alteration in the poly(A) site usage is suppressed by *pap1-2* mutation.

Northern blot of total RNA extracted from single and double mutant strains at the indicated times after the shift to cordycepin [20 μg/ml]. The membrane was hybridized with random-primed labelled probes against ACT1 mRNA.

2.10 Analysis of transcription termination

In the previous chapter we provide evidence that cordycepin affects poly(A) site recognition. Therefore, the possible involvement of genes, whose deletion causes cordycepin sensitivity, in termination was addressed by transcriptional run-on analysis (TRO) (Birse et al., 1998). TRO was performed with a plasmid borne *CYC1* gene that was under the control of a minimal *CUP1* promoter (pCUP-CYC1, figure 41).Distribution of run-on transcripts over single-stranded DNA probes (1–6 in figure 41) was analysed in wild-type and selected cordycepin sensitive mutant cells. Correct termination results in run-on signals over probes 1– 3 but not over probes 4–6 (Birse et al., 1998). Slot hybridisations showed that transcription termination was efficient in wild-type cells. The 38 bp deletion in the *CYC1* 3' UTR in the *CYC1-512* mutant causes a defect in termination and polyadenylation and the signals in probe 4 – 6 were strongly increased (Birse et al., 1998; Zaret and Sherman, 1982). The TRO results for all tested cordycepin sensitive mutants did not indicated a deficiency in termination of the pCUP-CYC1 gene (figure 41). RNA Pol II requires certain factors for a proper termination. Some of these factors are recruited to RNA Pol II already at the promoter. Therefore, the promoter might influence termination. TRO analysis was performed also with the GAL-CYC1 construct. However, the four tested gene deletion mutants did not display any termination defect (figure 42).

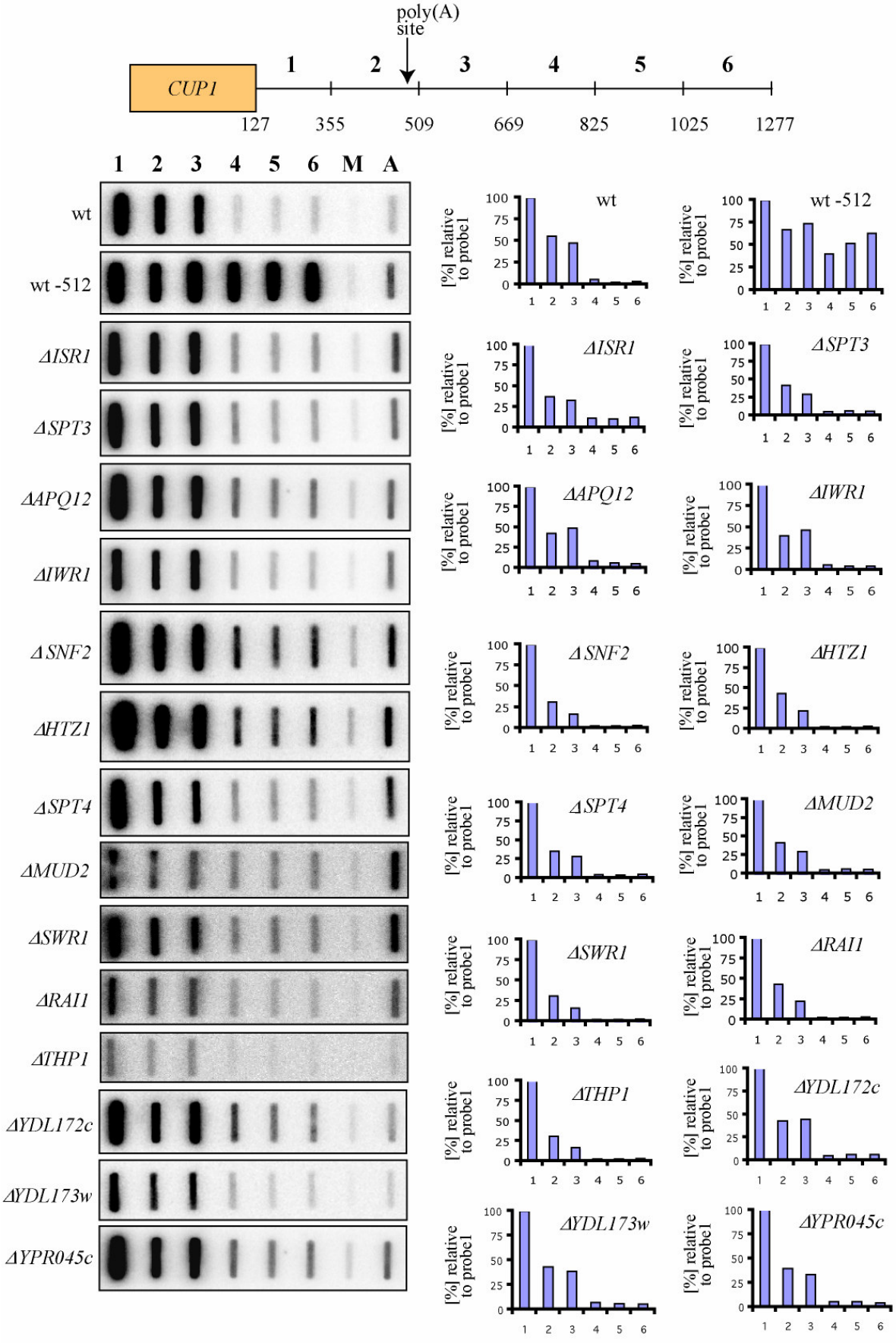
poly(A) site
CUP1
1
2
3
4
5
6
127
355
509
669
825
1025
1277
1 2 3 4 5 6 M A
wt
wt -512
ΔISR1
ΔSPT3
ΔAPQ12
ΔIWR1
ΔSNF2
ΔHTZ1
ΔSPT4
ΔMUD2
ΔSWR1
ΔRAI1
ΔTHP1
ΔYDL172c
ΔYDL173w
ΔYPR045c
[%] relative to probe1
100
75
50
25
0
1 2 3 4 5 6

Figure 41 Cordycepin sensitive gene deletion strains are not deficient in transcription termination.

A) *Representation of the pCUP-CYC1 gene that was analysed by transcriptional run-on (TRO). M13 probes (1–6) complementary to regions of the CYC1 gene (indicated in nucleotides relative to the transcription start site) and the poly(A) site are indicated.* ***B)*** *Slot blot hybridizations obtained following TRO indicated strains. 1– 6 represent CYC1 probes as described in (A), M13 (M) and ACT1 (A) probes served as controls.* ***C)*** *Quantification of run-on data. Values obtained by PhosphorImager scanning were corrected by subtraction of the M13 background signal and normalized to the value of probe 1, which was fixed at 100%.*

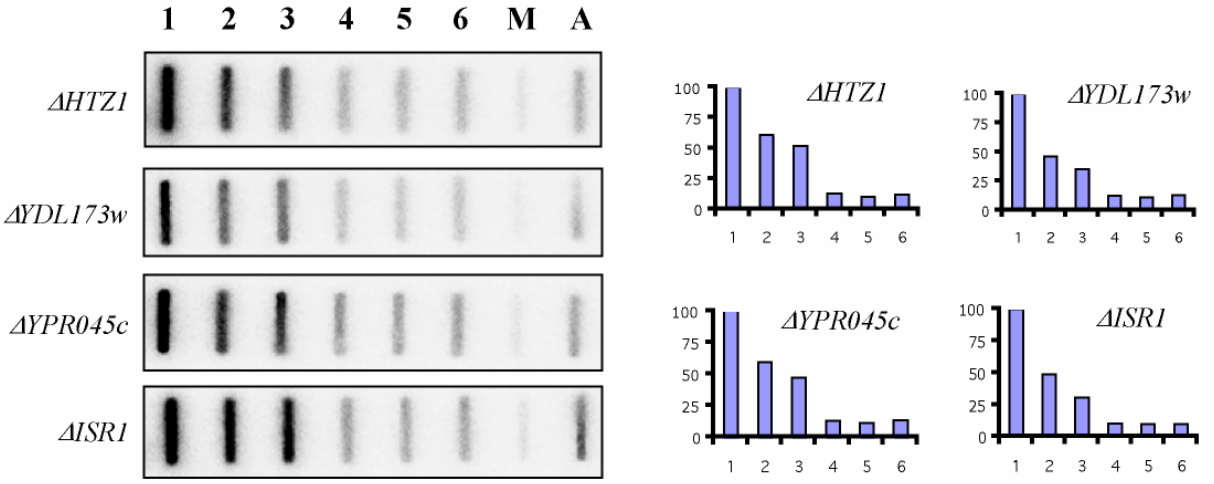

Figure 42 Transcriptional run-on with a GAL-CYC1 construct

Left: Slot blot hybridizations obtained following TRO indicated strains. 1– 6 represent CYC1 probes, M13 (M) and ACT1 (A) probes served as controls. Right: Quantification of run-on data. Values obtained by PhosphorImager scanning were corrected by subtraction of the M13 background signal and normalized to the value of probe 1, which was fixed at 100%.

Since elongated *CYH2* transcripts could be detected in Northern blots of wild-type cells after cordycepin addition (see part 3), TRO analysis was performed in wild-type cells after a shift to cordycepin containing media (figure 43). 30 and 60 minutes after cordycepin addition, no defect in transcription termination could be observed on the GAL-CYC1 gene, although at these time points the extended *CYH2* transcripts appeared. Cordycepin addition might therefore not lead to a general increase in read through transcripts and therefore to a less efficient termination. The appearance of the observed RNA transcripts of *CYH2* in the Northern blot might reflect a gene specific termination defect, which could not be resolved with TRO analysis on GAL-CYC1 gene. Cordycepin addition was also tested in the cordycepin resistant *pap1-2* mutant and as expected, no transcription termination defect could be observed.

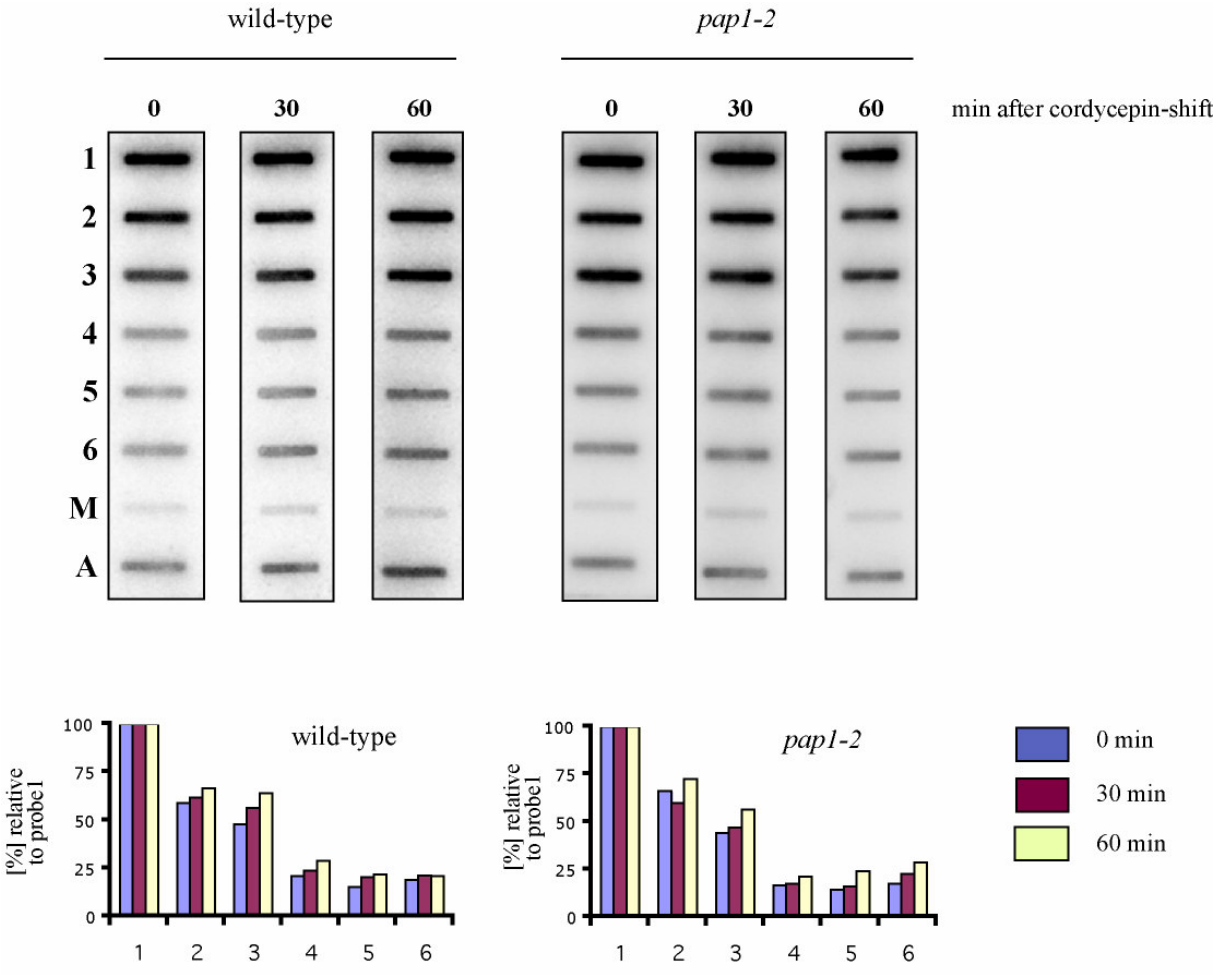

Figure 43 Cordycepin has no effect on transcription termination in wild-type and *pap1-1* mutant strains.

Slot blot hybridizations and quantification obtained following TRO of wild-type and pap1-2. The cells were shifted to 40 µg/ml cordycepin. At indicated time points the cells were harvested and TRO was performed.

3 Cordycepin-hypersensitive growth links elevated polyphosphate levels to inhibition of poly(A) polymerase in *Saccharomyces cerevisiae*

This chapter describes a project emerged from a collaboration with Florian Freimoser and Thomas Werner from the Insitute of plant science at the ETH of Zürich and the results have been published in Nucleic Acids Research 2008, Vol 36, Nr 2, 353 – 363.

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Cordycepin-hypersensitive growth links elevated polyphosphate levels to inhibition of poly(A) polymerase in *Saccharomyces cerevisiae*

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ABSTRACT

To identify genes involved in poly(A) metabolism, we screened the yeast gene deletion collection for growth defects in the presence of cordycepin (3′-deoxyadenosine), a precursor to the RNA chain terminating ATP analog cordycepin triphosphate. *Δpho80* and *Δpho85* strains, which have a constitutively active phosphate-response pathway, were identified as cordycepin hypersensitive. We show that inorganic polyphosphate (poly P) accumulated in these strains and that poly P is a potent inhibitor of poly(A) polymerase activity *in vitro*. Binding analyses of poly P and yeast Pap1p revealed an interaction with a k_D in the low nanomolar range. Poly P also bound mammalian poly(A) polymerase, however, with a 10-fold higher k_D compared to yeast Pap1p. Genetic tests with double mutants of *Δpho80* and other genes involved in phosphate homeostasis and poly P accumulation suggest that poly P contributed to cordycepin hypersensitivity. Synergistic inhibition of mRNA synthesis through poly P-mediated inhibition of Pap1p and through cordycepin-mediated RNA chain termination may thus account for hypersensitive growth of *Δpho80* and *Δpho85* strains in the presence of the chain terminator. Consistent with this, a mutation in the 3′-end formation component *rna14* was synthetic lethal in combination with *Δpho80*. Based on these observations, we suggest that binding of poly P to poly(A) polymerase negatively regulates its activity.

INTRODUCTION

Inorganic polyphosphate (poly P) comprises chains of 10s to 100s of phosphate residues, connected by energy-rich phospho-anhydride bonds. Despite the fact that poly P is an ubiquitous molecule detected in every living cell, our knowledge concerning its biochemistry and biological function remains incomplete (1,2). Poly P serves important roles as a substitute for ATP for sugar and adenylate kinases (3,4), as phosphate reservoir with osmotic advantage (5–8), as energy source (9) and reservoir (10), as buffer against alkaline stress (11) and as chelator of divalent ions (1).

In addition to these general functions, poly P has been implicated in a number of regulatory processes both in prokaryotes and eukaryotes. In *Escherichia coli*, poly P is essential for survival during stationary phase (12). It accumulates into large amounts in response to stress situations brought about by nutritional downshift from rich to minimal media (13), phosphate limitation (14), amino acid depletion (15) or nitrogen starvation. During the latter condition, poly P promotes ribosomal protein degradation by binding and activating the Lon protease in *E. coli* delivering amino acids needed to respond to starvation (16). Poly P is also ubiquitous in mammalian cells and tissues (17) where it has been implicated in a host of regulatory processes. For example, the mammalian TOR kinase, which is involved in cell growth and proliferation (18), is activated by poly P under conditions of nutritional starvation (19). This observation also led to the proposal of an evolutionary conserved role for poly P in stress response (19). Moreover, poly P was found to enhance proliferation of human fibroblast cells (20), to stimulate calcification of osteoblast-like cells (21),

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to inhibit the secretion of immunoglobulin and to stimulate apoptosis in human plasma and myeloma cells (22), and more recently, poly P has been shown to have anti-metastatic and anti-angiogenic activities (23).

Interestingly, there are several reports that link poly P to the regulation of gene expression through effects on RNA polymerase transcription. In *E. coli*, poly P is associated with RNA polymerase during the stationary phase and inhibits specifically the transcriptional activity of the enzymes associated with the $E\sigma^{70}$ promoter-recognition subunit, which is involved in the transcription of genes during exponential growth phase (24). Moreover, poly P induces transcription of *rpoS*, the stationary phase σ factor (25). Polyphosphate kinase (Ppk1), the enzyme synthesizing poly P, is a component of the *E. coli* RNA degradosome (26). Since poly P is a potent inhibitor of the degradosome, it was suggested that Ppk1 might act to maintain a correct microenvironment for proper mRNA degradation (26). In addition, poly P has been shown to associate with ribosomes and to suppress misincorporation of amino acids during translation (27).

In yeast, poly P concentrations can reach ~120 mM (1) and thus poly P contributes up to 20% of the cellular dry weight. Most of the poly P (90–99%) is localized to the vacuole (28,29), but poly P was also detected in the cytoplasm and the nuclei of yeast (30,33). For rat liver nuclei, micromolar poly P concentrations were reported (17) and for yeast nuclei a similar level of poly P is assumed (1). Poly P content of a yeast cell is strongly dependent on the growth phase (31,32) and the average length of nuclear poly P polymers of approximately 45 phosphate residues was found to be changing dynamically with growth conditions (33). Enzymes involved in eukaryotic poly P synthesis remain mostly elusive to this day (1), but activities indicative of an active poly P catabolism have been associated with all cellular compartments (31,34), including the nucleus (30,35). The observation that a double mutant of *PPN1* [encoding a endopolyphosphatase; (36)] and *PPX1* [encoding an exopolyphosphatase; (37)] rapidly looses viability in stationary phase (36), underscores the biological importance of poly P. We recently screened the entire collection of haploid yeast gene deletion mutants for poly P content (38) and found that poly P metabolism and primary metabolism (e.g. ATP and phosphate homeostasis) were strongly interdependent. This screen also revealed that all cellular compartments are linked to poly P homeostasis (38).

In this work, we establish a connection between cellular accumulation of poly P and inhibition of poly(A) polymerase activity. Yeast strains mutant in central components of the phosphate response pathway (*Δpho80* and *Δpho85*) contained increased amounts of poly P that contributed to cordycepin-hypersensitive growth. We propose a role for poly P in negatively regulating polyadenylation through inhibition of poly(A) polymerase.

MATERIALS AND METHODS

Yeast strains and plasmids

Wild-type BY4741 (*Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and isogenic mutant strains were obtained from EUROSCARF. The *rna14-1* genotype is *Mat a*; *ura3-1*; *trp1-1*; *ade2-1*; *leu2-3*; *112*; *his3-11*; *15*; *rna14-1* (39). Double mutants were generated by disrupting the *PHO80* open reading frame with a *NatR* cassette by homologous recombination in *Δpho4*, *Δpho2*, *Δpho84 and rna14-1* strains. *PHO80* and *PHO85* genes were cloned into pRS313 using BamHI and NotI restriction sites following PCR amplification from genomic DNA with primers PHO80-5′ AAGATCGGATCCCTTTCTATGGAAATA TGAATG and PHO80-3′ GATCTTGCGGCCGCAAAG AACAGTGATGATATGAAT and PHO85-5′ AAGAT CGGATCCTGTTTTAGAAATATGTGCACT and PHO85-3′ GATCTTGCGGCCGCTTTACGTTCTGCT CTCTCACTT. Yeast strains were grown at 30°C either in YPD (1% yeast extract, 2% tryptone, 2% glucose) or in synthetic complete medium (SD, yeast nitrogen base and complete amino acid mixture plus 2% glucose). SD was supplemented with cordycepin (40 μg/ml) purchased from Sigma or 5′-Fluoroorotic acid (1 mg/ml, Zymo Research, Orange, CA, USA).

Poly(A) length analysis

Assays were performed essentially as described (40). In the standard reaction, 2 μg of total RNA was incubated with 400 ng recombinantly expressed yeast poly(A) polymerase (a gift from G. Martin, Basel) and 0.2 μl [α-^{32}P]-cordycepin triphosphate (Perkin–Elmer) in reaction buffer (20 mM Tris–HCl pH 7.0, 50 mM KCl, 0.7 mM $MnCl_2$, 10% Glycerol, 100 μg/ml BSA) for 30 min at 30°C in a total volume of 12 μl. After heat inactivation, RNA was digested with RNase A and RNase T1, followed by proteinase K treatment. Precipitated RNAs were resolved on 15%/8.3 M urea polyacrylamide gels, that were exposed and visualized on a FLA-7000 phosphor-imager (Fuji).

Polyadenylation assays

Conditions were as described in Refs (41) and (42) with modifications. The yeast poly(A) polymerase reaction mixture of 15 μl contained 100 ng yeast poly(A) polymerase (a generous gift from G. Martin, Basel), 5 pmol of 5′-end labeled A_{15} RNA primer, 0.5 mM ATP, 5 mM $MgCl_2$, 25 mM Tris–HCl pH 7.9, 20 mM KCl, 10% glycerol, 0.01 mM EDTA, 0.1 mg/ml BSA, 1 mM DTT and 0.02% Nonidet P-40. The reaction mixture with bovine poly(A) polymerase consisted of 200 ng bovine poly(A) polymerase (a gift from G. Martin, Basel), 5 pmol of 5′ end-labeled A_{15} RNA primer, 0.5 mM ATP, 25 mM Tris–HCl pH 8.3, 40 mM KCl, 6 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM DTT, 0.01% Nonidet P-40, 10% glycerol and 200 μg/ml BSA in a total volume of 15 μl. Poly P was added to the reactions as indicated in the figure legends.

Reactions were incubated at 30°C for the indicated time and stopped by addition of 25 mM EDTA. Reaction products were precipitated and resolved on 15%/8.3 M urea polyacrylamide gels that were exposed and visualized on a FLA-7000 phosphor-imager (Fuji).

Purification of recombinant *E. coli* polyphosphate kinase

The gene encoding the *E. coli* polyphosphate kinase [EcPPK; (43)] was amplified with the primers CATGCCATGGGTCAGGAAAAGCTATACATCG and CGCGGATCCTGCGGACGAGGGGATTTATCG and cloned with the NcoI and BamHI sites (underlined) into the expression vector pETM41 (EMBL Protein Expression and Purification Unit). EcPPK was expressed as a fusion with a maltose-binding protein in *E. coli* BL21 cells and purified on amylose resin following standard protocols (New England BioLabs, Beverly, MA, USA). The activity was determined with the reverse reaction by measuring ATP synthesis at 37°C in 50 µl reactions containing 10 µM poly P_{88}, 1 µM ADP, 50 mM Tris and 50 mM malate, pH 6.5 and purified EcPPK. The reactions were stopped by heat inactivation at 90°C for 2 min. ATP was quantified after the addition of 50 µl luciferase reaction mixture (ATP bioluminescence assay kit CLS II, Roche Molecular Biochemicals) in a luminometer [Lumat LB 9507 (Berthold Technologies GmbH & Co. KG)].

Poly P measurements and poly P synthesis *in vitro*

Determination of poly P concentration was performed as previously described (32). Poly P with an average chain length of 750 residues was synthesized as described (13) except for some modifications. Poly P was synthesized in 200 µl reactions containing 138 nM [γ-^{32}P]-ATP, 1 mM ATP, 2 mM creatine-phosphate, 6 U creatine phosphokinase (from rabbit muscle, Sigma-Aldrich), 150 U polyphosphate kinase (1 unit corresponds to the transfer of 1 pmol P_i to ADP/min), 5 mM $MgCl_2$, 50 mM Tris and 50 mM malate (pH 6.5). The reactions were incubated for 16 h at 30°C. ^{32}P-labeled poly P was purified as described (33) and was eluted in 100 µl water. Radioactivity was measured in a scintillation counter (LS1801, Beckman Instruments, Fullerton, California) and the poly P concentration was calculated based on the fraction of ^{32}P that was incorporated in poly P (resulting typically in ~3 mM considering phosphate residues).

Poly P binding

Poly P binding assays were performed as reported earlier (16). Purified poly(A) polymerase from *Saccharomyces cerevisiae* and *Bos taurus* was diluted to a concentration of 200 nM in 100 µl reaction buffer (50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$). Equal volumes of radioactive poly P_{750} (0.3–30 nM, 890 c.p.m./pmol for yeast and 132 000 c.p.m./pmol for bovine poly(A) polymerase) were added. After 5 min at 37°C, the mixtures were applied to nitrocellulose filter discs (0.45 µm) and washed twice with 1 ml ice cold TBS (50 mM Tris–HCl pH 7.5, 100 mM NaCl). The remaining radioactivity on the filters, corresponding to the amount of poly P–protein complex, was measured in a scintillation counter.

RESULTS

Δpho85 and *Δpho80* strains are hypersensitive towards cordycepin

Poly(A) addition by poly(A) polymerase is terminated *in vitro* in the presence of cordycepin triphosphate (CoTP) (44,45). Since temperature-sensitive mutations in the essential 3′ end formation factors Rna14p and Rna15p render yeast cells sensitive to cordycepin (3′-deoxyadenosine) (46), we reasoned that a growth phenotype in the presence of this drug may identify mutant strains involved in poly(A) metabolism. Cordycepin is taken up by the yeast (47) and converted into the RNA-chain terminating CoTP, which is a substrate for RNA synthesis. The presence of 40 µg/ml cordycepin in the medium has only mild toxic effects on wild-type cells and increased the doubling time in liquid culture by ~20% (data not shown). In contrast, a strain lacking the 3′ end formation factor Ref2p (48) showed severe growth inhibition (Figure 1A). Thus, strains lacking non-essential genes involved in poly(A) metabolism can be identified through cordycepin-hypersensitive growth.

We screened a haploid *S. cerevisiae* gene deletion collection (49) for growth defects in the presence of cordycepin using a simple drop test on agar plates. The entire results of this screening will be presented elsewhere (S.H. and B.D., unpublished data). Here we focus on the further characterization of cordycepin-hypersensitive growth observed with *Δpho85* and *Δpho80* strains (Figure 1A). Complementation of the mutant strains with plasmids carrying wild-type *PHO85* and *PHO80* genes, respectively, demonstrated a direct requirement for these genes for cordycepin resistance. Empty plasmids, in contrast, did not complement. Pho85p kinase and its cyclin partner Pho80p form a complex and are central regulators of the *PHO* pathway in yeast (Figure 1B) (50). However, Pho85p can associate with nine other cyclins to perform functions in various cellular pathways including the cell cycle, carbon-source utilization and glycogen metabolism (51). We also tested mutant strains of seven other Pho85p cyclins (Figure 1C; *Δpcl1*, *Δpcl2*, *Δpcl6*, *Δpcl7*, *Δpcl8*, *Δpcl9* and *Δclg1*). Since we could not observe sensitivity with these strains, the cordycepin-hypersensitive growth of *Δpho85* and *Δpho80* appeared to be mainly linked to their function in phosphate homeostasis. The absence of Pho85p or Pho80p results in constitutively active transcription of phosphate-dependent genes (50). To evaluate the relation of other *PHO* pathway components and the observed cordycepin sensitivity, we analyzed strains lacking the transcription factors Pho4p and Pho2p, the cyclin-dependent kinase inhibitor Pho81p, the high-affinity phosphate transporter Pho84p and the target gene *PHO5* (encoding a secreted acid

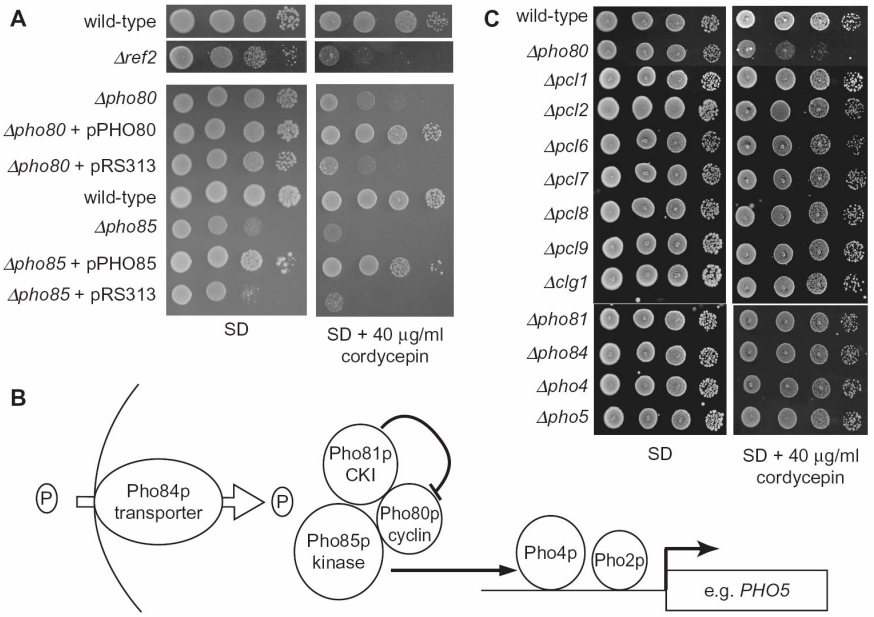

Figure 1. *Δpho85* and *Δpho80* strains display cordycepin-hypersensitive growth. (**A**) Twenty-fold serial dilutions of the indicated strains were spotted on agar plates that either lacked (SD) or contained 40 μg/ml cordycepin; plates were photographed after 72 h of incubation at 30°C. Complementation of *Δpho80* and *Δpho85* phenotypes was tested with empty plasmid (pRS313) or with the same vector carrying the *PHO80* or *PHO85* genes, respectively. (**B**) Schematic representation of key components of the *PHO* pathway (50). Pho84p is a high-affinity phosphate transporter, Pho85p and Pho80p form a cyclin-CDK (cyclin-dependent kinase) pair and Pho81p is an associated CDK inhibitor; the Pho4p transcription factor (the target of the Pho85p/Pho80p complex) in its hypo-phosphorylated form associates with the homeodomain transcription factor Pho2p to drive the expression of phosphate-regulated genes, like e.g. the Pho5p secreted acid phosphatase. (**C**) Cordycepin hypersensitivity is restricted to *PHO* pathway-dependent functions of Pho85p. Drop test as in A) with mutant yeast strains lacking the indicated Pho85p cyclins (upper panel) and components of the *PHO* pathway (lower panel) on medium lacking or containing 40 μg/ml cordycepin.

phosphatase). None of these strains reacted to the drug (Figure 1C and data not shown). We conclude that a constitutively active *PHO* pathway underlies the observed cordycepin hypersensitivity of *Δpho85* and *Δpho80* mutants.

Δpho85 and *Δpho80* strains accumulate an inhibitor of poly(A) polymerase

Since CoTP can act as a terminator of the poly(A) addition reaction *in vitro* (44,45), we considered the possibility that cordycepin hypersensitivity of *Δpho85* and *Δpho80* strains may relate to this process. Therefore, we isolated total RNA from wild-type and mutant strains and analyzed the length distribution of cellular poly(A). The protocol for poly(A) labeling that we employed included the transfer of radioactively labeled [α^{32}P]-CoTP to 3′-OH groups present in the RNA preparation with recombinant yeast poly(A) polymerase (40). With wild-type RNA, a uniformly distributed length of approximately 10–70 adenosines was observed (Figure 2A). Quite unexpectedly, we were unable to label any poly(A) isolated from *Δpho85* and *Δpho80* strains under standard assay conditions (i.e. 1–2 μg of total RNA; Figure 2A). We considered the possibility that some inhibitory activity may be present in the RNA preparations of the mutant strains interfering with the activity of poly(A) polymerase and performed the labeling assay with decreasing amounts of total RNA. We observed that a 4- to 8-fold dilution of the total RNA from *Δpho85* and *Δpho80* strains indeed allowed poly(A) labeling (Figure 2A). Furthermore, reduced amounts of wild-type RNA gave increased labeling efficiency. We conclude that an inhibitory activity was present in the total RNA preparations from *Δpho85* and *Δpho80* strains interfering with the poly(A) labeling test and that the same inhibitor was also present in wild-type RNA, albeit at lower concentration.

Poly P accumulates in *Δpho85* and *Δpho80* strains and interferes with RNA 3′ end labeling by poly(A) polymerase

We hypothesized that the co-purification of a metabolite with *Δpho85* and *Δpho80* RNA may cause the inhibition of the poly(A) labeling reaction. It was suggested that the *PHO* pathway is involved in regulating the synthesis of poly P (6). However, inconsistent results were obtained when *Δpho85* and *Δpho80* strains were previously analyzed for poly P contents (6,52). Therefore, we measured poly P levels in wild-type and mutant strains with a recently developed assay (32) and found that *Δpho85* and *Δpho80* strains had approximately 3-fold more poly P than wild type (Figure 2B). In contrast,

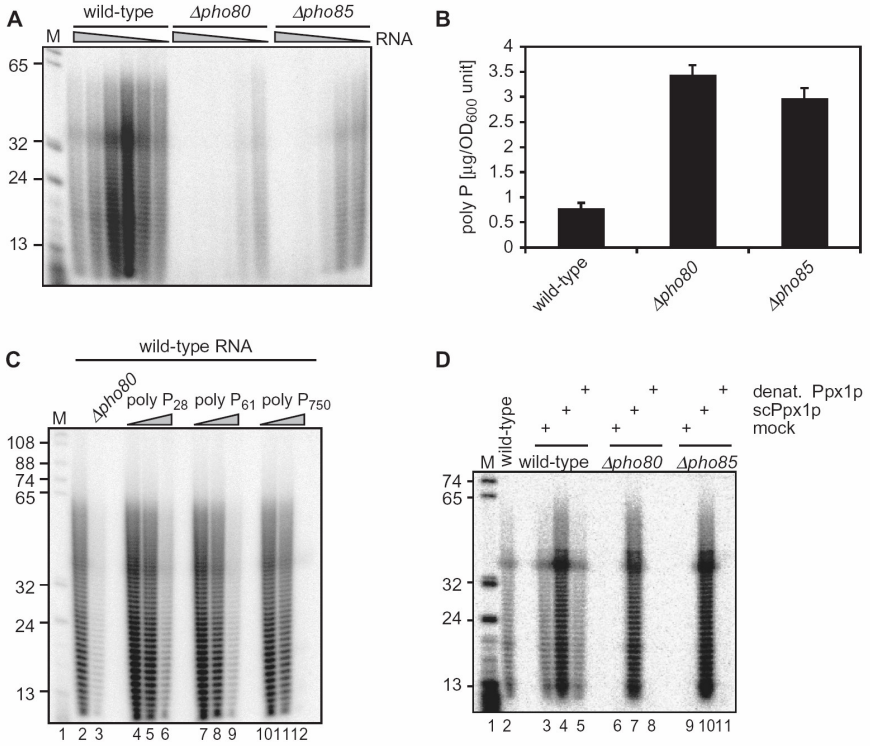

Figure 2. *Δpho85* and *Δpho80* strains accumulate poly P, an inhibitor of poly(A) polymerase. (**A**) Poly(A)-labeling assays with total RNA extracted from wild-type, *Δpho80* and *Δpho85* strains. Decreasing amounts of total RNA (2 µg, 1 µg, 0.5 µg, 250 ng, 125 ng and 75 ng) were included with recombinant yeast Pap1p in the reactions. Following RNase A/T1 digestion, the labeled poly(A) tails were loaded on a 15% denaturing polyacrylamide gel. Poly(A) labeling was observed in *Δpho80* and *Δpho85* only following dilution of the RNA included in the reactions. HpaI-digested pBR322 fragments were 5′-end labeled and served as marker bands (M) with the indicated size. (**B**) Poly P contents in wild-type, *Δpho80* and *Δpho85* strains following growth in YPD medium for 6 h. (**C**) Poly(A) labeling assay as described in (A) with the addition of *Δpho80* RNA (lane 3) or of increasing amounts of poly P (60 nM to 6 µM poly P_{28}; 27 nM to 2.7 µM poly P_{61}; and 2 nM to 200 nM poly P_{750}) with the indicated average chain length (lanes 4–12) to poly(A)-labeling assays with wild-type RNA. Lane 2 shows the reaction of wild-type RNA without addition of inhibitors. (**D**) Poly(A)-labeling assay as described in (A) with total RNA obtained from wild-type, *Δpho80* and *Δpho85* strains. RNAs were treated as indicated with yeast exopolyphosphatase (scPpx1p), with heat-denatured exopolyphosphase (denat. Ppx1p) or buffer (mock) before the labeling reaction was performed.

mutants in other components of the *PHO* pathway did not show this accumulation; indeed poly P concentrations were reduced in *Δpho4*, *Δpho2* and *Δpho84* mutant strains (Figure 5D) (6,53). These results support the idea that poly P metabolism is regulated by the *PHO* pathway and that constitutive activity of this pathway (in the absence of Pho85p and Pho80p) resulted in increased cellular poly P concentrations.

The accumulation of poly P in *Δpho85* and *Δpho80* strains prompted us to test whether poly P is an inhibitor of poly(A) polymerase. Therefore, we repeated the poly(A) labeling test with standard amounts of RNA (2 µg) and added either *Δpho80* RNA or increasing amounts of poly P with different average chain length (21, 61 and 750 phosphate residues). We observed that both the addition of *Δpho80* RNA and of poly P resulted in inhibition of the labeling reaction (Figure 2C). The inhibition was nearly complete with the highest concentration of poly P employed (200 nM). However, in multiple repetitions of this experiment, we could not observe a clear correlation of the degree of inhibition and the chain length of poly P included in the assay.

To demonstrate that poly P is indeed the inhibitory activity present in the *Δpho85* and *Δpho80* RNA preparations, we treated the RNAs with recombinant yeast exopolyphosphatase (Ppx1p) prior to the labeling reaction. Ppx1p specifically degrades poly P and does not act on pyrophosphate or ATP (54). Figure 2D shows that we were able to label the poly(A) content of *Δpho85* and *Δpho80* RNA preparations following Ppx1p treatment and the observed distribution of poly(A) in the mutant strains appeared normal. No poly(A) was detected when reaction buffer replaced Ppx1p or when the Ppx1p was heat-denatured prior to use. Interestingly, the labeling efficiency of wild-type RNA following

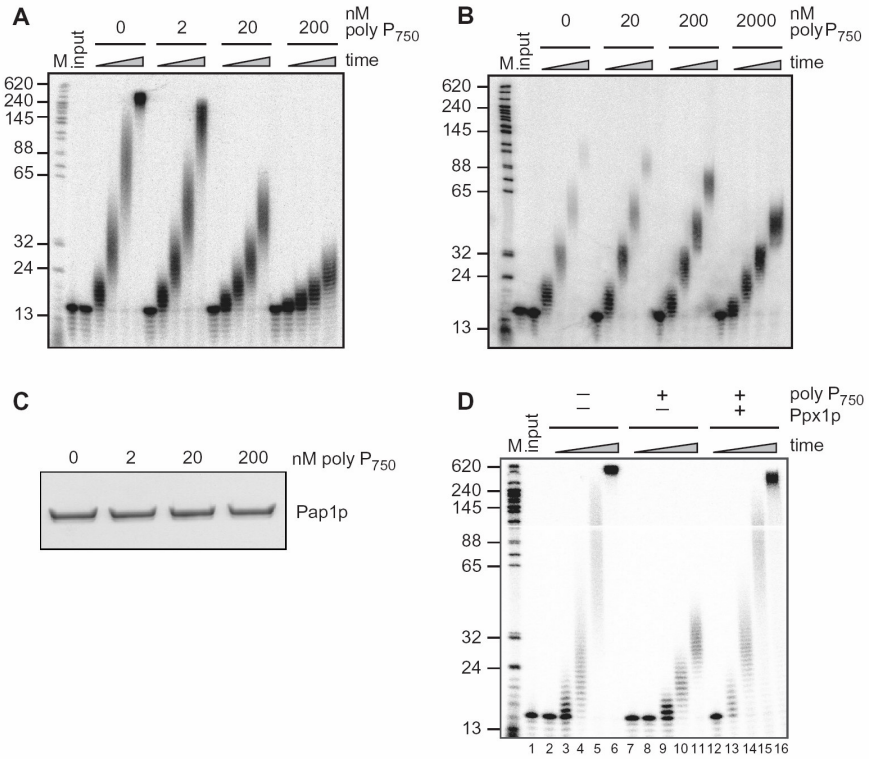

Figure 3. Poly P inhibition of poly(A) polymerase activity *in vitro*. (**A**) ^{32}P-labeled A_{15} RNA primer was incubated with poly(A) polymerase and cold ATP in the absence or presence of the indicated amounts of poly P_{750}. After 0, 5, 15, 30 and 60 min incubation time, an aliquot of the reaction was removed and analyzed on a 15% denaturing polyacrylamide gel. (**A**) Reaction with *S. cerevisiae* Pap1p and (**B**) reaction with bovine poly(A) polymerase. (**C**) Effects of poly P binding on the solubility of Pap1p. Following incubation of Pap1p with the indicated amounts of poly P_{750}, reactions were cleared by centrifugation and soluble material was resolved by denaturing SDS–PAGE and stained with Coomassie Brilliant Blue. (**D**) Polyadenylation assay as described in (A). However, Pap1p was pre-incubated with H_2O (lanes 2–6) or 200 nM poly P_{750} (lanes 7–16) for 15 min at 30°C. Subsequently, reaction mixtures were treated with H_2O (lanes 2–11) or Ppx1p (lanes 12–16) for 30 min at 30°C and polyadenylation reactions were then started by the addition of A_{15} primer. HpaI-digested pBR322 fragments were 5′-end labeled and served as marker bands (M) with the indicated size.

Ppx1p pre-treatment was also enhanced, demonstrating the presence of poly P also in wild-type RNA, consistent with our poly P measurements in wild-type strains (Figure 2B). Taken together the results from this figure show that poly P accumulated in *Δpho85* and *Δpho80* strains, that this metabolite co-purified with total RNA extracted from mutant and wild-type strains and that poly P was responsible for the observed inhibition of poly(A) polymerase.

Poly P binds poly(A) polymerase

Since the poly(A)-labeling assay uses CoTP, poly(A) polymerase activity is restricted to a single round of nucleoside addition. To examine the effects of poly P under multiple turn-over conditions, we performed poly(A) polymerase assays with an end-labeled A_{15} RNA primer, cold ATP and recombinant yeast Pap1p (Figure 3A). Under the conditions employed, yeast Pap1p extended the primer to ~300 to 400 adenosines in 60 min. In the presence of 2 nM poly P_{750}, we observed a slight reduction of the length of the polyadenylation products during the time course. This effect was stronger with 20 nM poly P_{750} and 200 nM resulted in almost complete inhibition. Similar effects were observed when poly P of varying length (average of 28 and 61 phosphate residues) was tested (data not shown). The poly P concentrations used for these assays are likely to be physiologically relevant, as yeast nuclei are thought to harbor poly P concentrations of up to 89 μM (1).

Next, we tested whether poly P also inhibited the activity of bovine poly(A) polymerase. Under the employed conditions bovine Pap extended the A_{15} RNA primer by approximately 100 adenosines within 1 hour (Figure 3B). When 20 nM to 2 μM of poly P_{750} were included in the reaction, the length of the products was reduced but inhibition was not complete. Therefore, poly P was a less potent inhibitor of bovine poly(A) polymerase compared to the yeast enzyme.

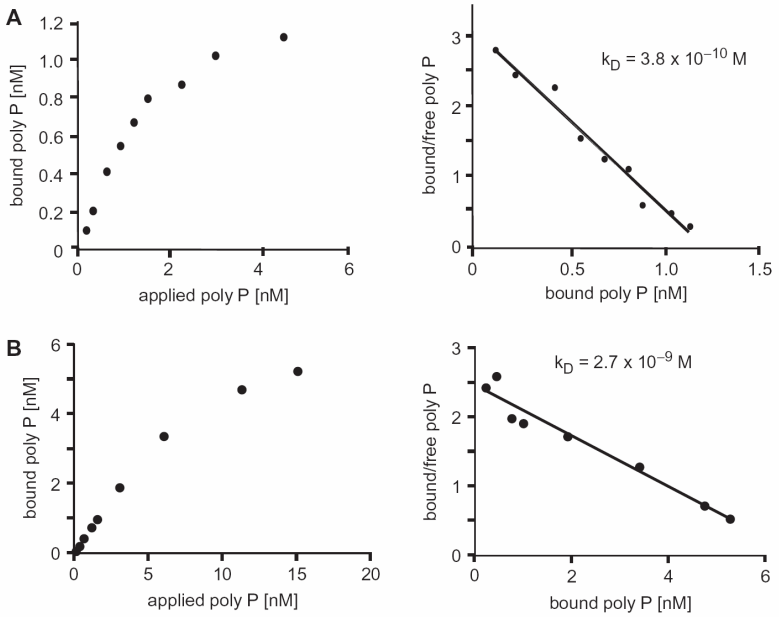

Figure 4. Binding of poly P to yeast and bovine poly(A) polymerase. Poly P binding by yeast (**A**) and bovine (**B**) poly(A) polymerase determined by filter-binding assay as a function of poly P_{750} concentration (left panels). Scatchard plot representations of the obtained data (right panels) were used to derive the indicated apparent k_D values. The slope of the lines obtained equal $-1/k_D$.

Poly P is an efficient chelator of divalent metal ions (1). A possible explanation for the inhibitory effect on poly(A) polymerase activity could be the chelation of magnesium ions which are essential for Pap1p function. However, the magnesium in our assays is present in large molar excess over inhibitory poly P concentrations (calculated as the amount of free inorganic phosphate). Therefore, we considered the possibility that poly P may inhibit the enzymes by direct binding. To test this, we performed filter-binding assays with radioactively labeled poly P_{750} and yeast or bovine poly(A) polymerase. We determined an apparent dissociation constant of 0.38 nM for the yeast enzyme and of 2.7 nM for the bovine enzyme (Figure 4). It has to be pointed out, however, that one poly P molecule is likely to provide multiple binding sites for Pap1p. Therefore, it seems possible that binding was influenced by avidity effects. Irrespective of that, the tight binding of poly P to poly(A) polymerase was most likely responsible for the observed inhibitory effects of poly P in polyadenylation assays.

To address the mechanisms of inhibition, we considered the possibility that poly P binding may result in unspecific aggregation or precipitation of the enzyme. To test this idea we incubated Pap1p with increasing amounts of poly P, collected protein aggregates by centrifugation and resolved remaining soluble material by SDS–PAGE. As shown in Figure 3C, poly P binding did not result in precipitation of the enzyme. Thus, unspecific aggregation can be excluded as a possible reason for the observed inhibition. To test whether the inhibition of poly P on Pap1p activity was reversible, we pre-incubated Pap1p with 200 nM poly P to allow binding of poly P and Pap1p. As expected, this resulted in strong inhibition of polyadenylation activity (Figure 3D, lanes 7–11). However, Pap1p activity could be almost completely restored by treatment of the Pap1p–poly P complexes with Ppx1p (Figure 3D, lanes 12–16). This demonstrated that the inhibition of Pap1p by poly P was reversible.

The Pap1p that we used in the poly(A) length analysis and in the polyadenylation assay was purified from *E. coli*, which also contains poly P (1). Thus, purified Pap1p may already be partially bound to poly P causing a partial inhibition of the enzymatic activity. To test this, we performed a polyadenylation assays with Pap1p that was pretreated with Ppx1p. Since the treatment did not noticeably increase the activity of Pap1p preparation (data not shown), we conclude that most of Pap1p that we used in our experiments was not pre-bound by poly P. This indicated that the conditions used for over-expression of Pap1p did not induce significant poly P accumulation in *E. coli*.

Poly P accumulation contributes to cordycepin sensitivity

We showed that *Δpho85* and *Δpho80* strains are cordycepin hypersensitive, that these strains accumulated poly P and that poly P acted as an efficient inhibitor of poly(A) polymerase. These observations suggested a

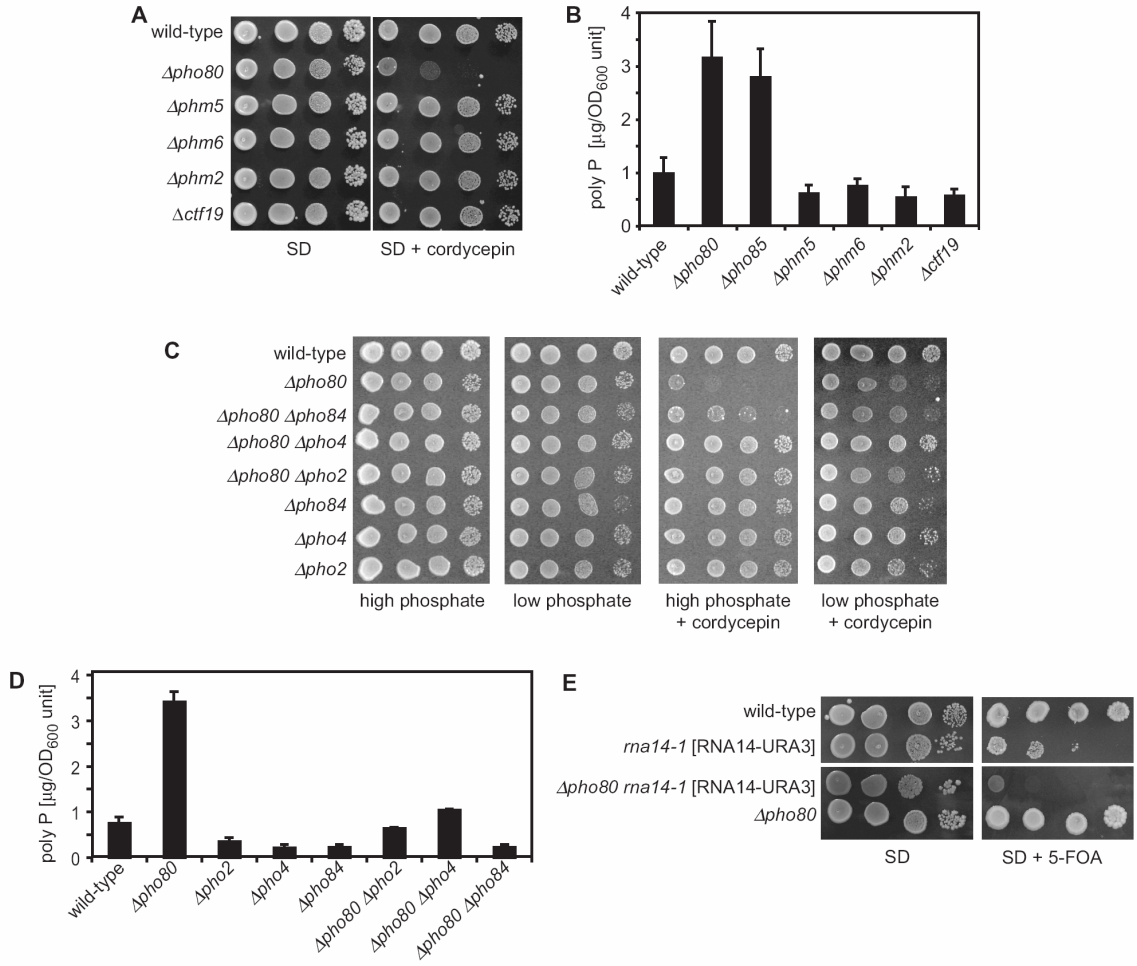

Figure 5. Cellular poly P contents correlates with cordycepin-hypersensitive growth. (**A**) Drop test as described in Figure 1A with the indicated strains on agar plates containing or lacking cordycepin (40 µg/ml) as indicated. Plates were incubated for 72 h at 30°C. (**B**) Poly P content of the indicated strains was determined following growth in YPD medium for 6 h as described. (**C**) Drop test of 20-fold serial dilutions of the indicated strains on agar plates containing 10 mM (high phosphate) or 0.1 mM (low phosphate) phosphate and cordycepin (40 µg/ml) as indicated. Plates were incubated for 72 h at 30°C. (**D**) Poly P content of the indicated strains was determined following growth in YPD medium for 6 h. (**E**) The *Δpho80* mutation is synthetic lethal in combination with *rna14-1*. The *PHO80* gene was deleted in a *rna14-1* strain that carried a plasmid with wild-type *RNA14* and the *URA3* marker gene. Serial dilutions of indicated wild-type, single and double mutant strains were spotted on synthetic complete medium lacking (SD) or containing the drug 5-Fluoroortic Acid (SD + 5-FOA) and incubated for 72 h at 30°C. Since 5-FOA selects against the *URA3* marked plasmid, lack of growth in the presence of the drug indicated that cell viability was dependent on the wild-type copy of *RNA14* present on the plasmid.

correlation between cellular poly P accumulation and the drug-dependent growth defect. Ogawa and co-workers (6) reported accumulation of poly P in strains mutant for *PHM5*, *PHM6*, *PHM2* and *CTF19* during the so-called 'overplus' conditions. However, we could not observe cordycepin-hypersensitive growth associated with these mutant strains (Figure 5A). Moreover, our poly P measurements in these strains did not indicate a significant increase of the polymer under conditions, which revealed a strong increase in *Δpho85* and *Δpho80* strains (Figure 5B). The differences between the measurements presented here and in Ogawa *et al.* are possibly due to differences in growth conditions at the time of measurement. Furthermore, our recent genome-wide assessment of poly P metabolism failed to identify other mutants that would result in poly P accumulation comparable to levels observed in *Δpho85* and *Δpho80* strains (38). It seems possible, therefore, that cordycepin-hypersensitive growth is a consequence of elevated poly P levels exceeding a certain threshold in *Δpho85* and *Δpho80* strains and that such levels are not achieved or stably maintained in other mutants defective in poly P metabolism.

To further test whether cordycepin sensitivity was due to poly P accumulation in *Δpho85* and *Δpho80* mutants, we interfered with the ability of the strains to synthesize poly P and asked whether this alleviated cordycepin sensitivity. When low concentrations of phosphate (0.1 mM) are present in the medium growth of the yeast is only marginally reduced, but no significant poly P accumulation can be measured (32). We tested *Δpho80* strains on plates containing cordycepin but low phosphate and found that *Δpho80* strains showed significant resistance to cordycepin under these conditions (Figure 5C). This observation suggested that abundant phosphate was required for the observed cordycepin hypersensitivity. Furthermore, the *PHO* pathway regulates poly P accumulation in yeast (6). Figure 5D shows that cellular levels of poly P were significantly reduced in *Δpho2*, *Δpho4* and *Δpho84* mutants compared to wild type. We produced double mutants of *Δpho80* and other components of the *PHO* pathway and found that *Δpho80 Δpho4*, *Δpho80 Δpho2* and *Δpho80 Δpho84* double mutants did not accumulate poly P (Figure 5D). The cellular poly P content of the analyzed double mutants correlated well with a loss of cordycepin hypersensitivity associated with the single *Δpho80* mutation (Figure 5C). These observations are consistent with the proposal that poly P accumulation in *Δpho80* strains contributed to cordycepin hypersensitivity. Increased cellular poly P levels may interfere with poly(A) polymerase activity, and this may cause hypersensitive growth in the presence of cordycepin, another inhibitor of RNA synthesis.

To further test this idea we asked whether a temperature-sensitive mutation in the 3′ end formation factor Rna14p (*rna14-1*) can duplicate the effects observed with cordycepin in *Δpho80* mutants. It was previously shown that combination of mutations in *rna14* and *pap1* resulted in a synthetic growth defect (39) and *rna14* mutant strains are cordycepin hypersensitive [(46); our unpublished data]. Figure 5E shows that combination of the *Δpho80* mutation with the *rna14-1* allele indeed resulted in synthetic lethality. This genetic interaction strongly supported the proposal that accumulation of poly P in *Δpho80* strains affected 3′ end formation via inhibition of poly(A) polymerase activity and that this inhibition in combination with another defect in 3′ end formation led to the observed synthetic growth defect.

DISCUSSION

We provide evidence for an unexpected link between poly P metabolism and polyadenylation in yeast. We propose that a synergistic effect of poly P-mediated inhibition of poly(A) polymerase together with inhibition of RNA synthesis by CoTP can cause lethality. Synthetic lethality observed between *PHO80* and an essential factor involved in 3′ end formation underscores this assumption. Poly P may therefore play a role in the regulation of poly(A) polymerase activity.

The starting point of this work was an unbiased genome-wide screen for mutants that showed hypersensitive and/or resistant growth in the presence of cordycepin. The rational for this screen was that cordycepin will be converted to CoTP, which will then provoke RNA-chain termination in the nucleus. Although CoTP will terminate RNA synthesis when incorporated during transcription, it seems possible that polyadenylation would be a more sensitive target since up to 70 consecutive adenosines are incorporated during this process. This may be particularly pronounced under the low cordycepin conditions (40 μg/ml) that were employed during our screening; as expected, mutants in 3′ end formation factors displayed hypersensitive growth in the presence of such cordycepin concentrations (Figure 1A and data not shown). Although it remains unclear which other cellular ATP-consuming activities are sensitive to cordycepin or CoTP, cordycepin-hypersensitive growth can be indicative of a functional involvement of a mutation in 3′ end formation and poly(A) metabolism. A complete account of the results obtained from our cordycepin-dependent growth screening will be presented elsewhere (S.H. and B.D., unpublished data). However, no additional links emerged between cordycepin hypersensitivity and poly P metabolism other than the one presented here for *Δpho85* and *Δpho80* strains.

The kinase activity of the Pho85p/Pho80p CDK–cyclin complex controls phosphate-dependent gene expression by regulating both the activity and localization of the Pho4p transcription factor (50,55). Interestingly, poly P was proposed to act as a phosphate buffer that can be mobilized to maintain constant internal phosphate levels under conditions where external phosphate levels fluctuate (7,8) and the *PHO* pathway was linked to expression of genes that are involved in poly P homeostasis (6). As a consequence, constitutive activation of the *PHO* pathway (e.g. in *Δpho80* strains) was expected to result in increased poly P levels. However, Ogawa and co-workers (6) could not observe increased levels of poly P in *Δpho80* strains. In contrast, it was reported that *Δpho85* strains have increased poly P (52). We re-evaluated the poly P content of *Δpho85* and *Δpho80* strains and detected an approximately 3-fold increase in both strains compared to wild-type (Figure 2B). We attribute these conflicting poly P measurements to fluctuations of poly P levels in the yeast depending on the growth state and recently proposed a standardized procedure for poly P determination (32).

We found that the presence of increased amounts of co-purified poly P in RNA preparations from *Δpho85* and *Δpho80* strains was accompanied by a strong inhibition of poly(A) polymerase activity. Consistent with our observations, previous biochemical work on the effects of poly P on yeast Pap1p revealed an inhibitory activity on tRNA primed poly(A) synthesis (56). We show in this work by filter-binding assays that poly P directly binds to yeast Pap1p with a k_D in the low nanomolar range (0.38 nM). This value is comparable to the k_D of 0.48 nM that was determined for the poly P–Lon interaction, which stimulates Lon protease activity (16). Pap1p and Lon are thus the interactors with the highest reported affinities for poly P. Consistent with a physiological relevant role for the interaction of poly P and Pap1p, nuclear poly P levels were reported to reach micromolar concentrations (1); furthermore, nuclei predominantly contain

poly P of an average chain length of approximately 45 phosphate residues (33), and poly P with similar length (poly P_{28} and poly P_{61}) acted as efficient inhibitor of Pap1p in our *in vitro* experiments (Figure 2C). We have not delineated the binding site of poly P on Pap1p, but potential interaction surfaces include regions of RNA primer binding or of ATP binding (57,58). Notably, binding of poly P to bovine poly(A) polymerase and inhibition of its activity is approximately 10-fold weaker compared to the yeast enzyme. This may have important consequences on a potential *in vivo* interaction of poly P and poly(A) polymerases in higher eukaryotes.

What is the biological role of poly P-mediated inhibition of poly(A) polymerase activity? We predict that such a function must be connected to growth conditions that are accompanied by high poly P concentrations and that the control of poly P levels is at least partially under control of the *PHO* pathway. We recently showed that poly P content reaches a peak when cells experience the diauxic shift following depletion of glucose from the medium (32). This growth phase is characterized by major changes in the gene expression program (59,60). It seems possible that the down-regulation of many hundreds of genes during diauxic shift is accompanied by an inhibition of polyadenylation. In this context, it is worth pointing out that cellular poly(A) levels inversely correlate with poly P levels when cells enter diauxic shift (32,60). Our observation that the inhibitory effects of poly P on Pap1p were completely reversible (Figure 3D) may indicate that poly P action can be transient and controlled through a dynamic nuclear poly P metabolism (33). Furthermore, fluctuations of nuclear poly P levels may interfere with RNA processing in a microenvironment as was suggested for the regulation of the bacterial degradosome (26). Since no global polyadenylation defect could be observed at steady state in *Δpho80* and *Δpho85* strains (Figure 2D) poly P may act on specific gene loci and not as a general suppressor of Pap1p activity. A deeper understanding of poly P biochemistry and metabolism will be required to address these possibilities in future experiments.

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VII Discussion

Cordycepin is an adenosine analogue that has the ability to enter cellular nucleotide pools after its conversion into cordycepin triphosphate and its incorporation into RNA leads to termination of RNA synthesis. Importantly, CoTP is a biological active compound and its function as an antitumor antibiotic has been identified many years ago (Jagger et al., 1961). In case of the yeast RNA polymerases, presented kinetic parameters suggest that the inhibitory effect of cordycepin is higher on RNA Pol II than on poly(A) polymerase (Horowitz et al., 1976). However, the adenosine analogue revealed potent inhibitory activity on poly(A) polymerases of mammals (Müller et al., 1977), vaccina virus (Shuman and Moss, 1987) and of yeast (Hooker et al., 2001). Because cordycepin has been shown to act as a strong inhibitor of the enzyme terminal deoxynucleotidyl transferase (Müller et al., 1977) it is currently undergoing clinical trial phase I in the Unites States for treatment of TdT^+ leukaemia patients.

The molecular effects of cordycepin in *Saccharomyces cerevisiae*

The addition of cordycepin to yeast media exhibited several distinct effects on RNA metabolism. The drug produced a decrease in steady-state mRNA levels of several genes as expected for a chain terminator. The observed decrease seemed to correlate with the stability of the mRNA. *PGK1* has a half-live of over 90 minutes and *PGK1* mRNA levels remained stable for 120 minutes in presence of cordycepin. Furthermore, we detected extended RNA transcripts of *CYH2*, *ACT1* and *ASC1* mRNA. In the case of *ACT1* and *ASC1* mRNA, cordycepin changed the relative usage of the polyadenylation site in favour of the most distal poly(A) site. Mutant strains of factors of the 3' end machinery such as *RNA14*, *RNA15* and *YTH1* have been associated to a similar shift in poly(A) site usage (Mandart and Parker, 1995; Dichtl et al., 2002b). Most interestingly, also *pap1-1* exhibit a shift of poly(A) site usage (Mandart and Parker, 1995), which is unexpected because polyadenylation site selection precedes the process of poly(A) addition. We hypothesise that the binding of CoTP on Pap1p reduced effectiveness of the enzyme leading to the same phenotype as observed in the *pap1-1* mutant.

Strikingly, elongated transcripts of *CYH2* were detected, which were longer than 1 kb and unprocessed as they still contained the intron sequence. This effect of cordycepin was especially evident at lower concentrations (16 – 40 µg/ml) however at higher concentration (98 µg/ml) the decrease in mRNA levels was more prominent. The reason for the elongated *CYH2* transcripts could be a read-through of RNA Pol II induced by cordycepin by interfering with polyadenylation site recognition. Unspliced read-through transcripts are normally degraded through RNA surveillance mechanisms (see above) (Bousquet-Antonelli et al., 2000; LaCava et al., 2005). Using transcriptional run-on analysis, a defect of transcription termination in presence of cordycepin could not be detected in wild-type cells, which suggests that cordycepin does not cause a deficiency in transcription termination in general.

Consistent with our observations, cordycepin does not seem to affect only mRNA synthesis as a RNA chain terminator. It has been demonstrated before, that cordycepin is not a typical chemical inhibitor of transcription (Grigull et al., 2004). The authors found, that the gene expression profile obtained by cordycepin did not correlate well with those from transcriptional inhibitors such as 6-azauracil, 1,10-phenanthroline or the temperature-sensitive mutant strain *rpb1-1*.

Cordycepin as an inhibitor of RNA surveillance?

Gene expression profiles of wild-type cells revealed that more RNAs were increased more than twofold (222) in presence of the drug than decreased more than twofold (72). No gene ontology term was found to be significantly enriched in these RNA groups. Interestingly, 44% of the 222 increased RNAs were hORFs, which means that the fraction of hORFs almost doubled in comparison with the decreased RNA group (27.4%) or the RNAs obtained after processing the raw data (22.6%). The CUT *NEL025c* was stabilised in *ΔTRF4* and *ΔRRP6* mutants in presence of cordycepin and elongated *NEL025c* transcripts could be detected. CUTs are short, 300 – 600 nt, noncoding RNAs transcripts produced by RNA Pol II. They may represent 10% of intergenic transcripts in *S. cerevisiae* (Wyers et al., 2005). CUTs are polyadenylated through the TRAMP complex subunits Trf4p and Trf5p, which targets them for degradation by the nuclear exosome (Wyers et al., 2005). What could be the role of cordycepin in the CUT degradation pathway? CoTP might be incorporated into the destabilising poly(A) tail by the poly(A) polymerases Trf4p and

Trf5p resulting in a RNA chain termination and in shorter poly(A) tails. Since the poly(A) tail synthesised by the TRAMP complex is accelerating RNA decay, a shorter poly(A) tail might lead to a less efficient degradation by the nuclear exosome. Another possibility is that the incorporated CoMP at the 3' end of the poly(A) tail might block entry of the nuclear exosome. Maybe the combination of shorter poly(A) tails and a CoMP at the 3' end of the poly(A) tail led to the observed stabilisation of the CUT *NEL025c*. However, this effect is mild, since no stabilisation of *NEL025c* was observed in wild-type cells. But cordycepin enhanced the stabilisation of the CUT in a *ΔTRF4* or *ΔRRP6* deletion strain. CUTs have been implicated in the regulation of expression of neighbouring genes. For example, *IMD2* and *SER3* expression are regulated by transcription of a CUT (Kuehner and Brow, 2008; Martens et al., 2005). Thus, the stabilisation of CUT might have an effect on the expression of adjacent genes. Moreover, less efficient RNA surveillance could also lead to the detection of the extended *CYH2* transcripts in presence of cordycepin. These elongated transcripts are intron containing and not properly processed. They might be the product of a read-through by RNA Pol II and would probably be rapidly degraded by the TRAMP/exosome degradation pathway. It seems possible therefore, that in presence of the adenosine analogue, the degradation of these transcripts might be less efficient leading to stabilised *CYH2* transcripts that are detectable in Northern blot analysis.

Genome-wide identification of cordycepin targets

Determining the mode of action of a compound is a central issue in molecular medicine. Yeast is one of the simplest eukaryotic organisms. The single cell organism has a short life cycle of 90 minutes and is inexpensive to grow and maintain. Its haploid genome has a small size and is of relatively low complexity. Yeast has become a prominent model for human diseases and pathways, since at last 31% of the proteins encoded in the yeast genome have a human orthologue and nearly 50% of human disease genes exhibit yeast orthologes (Foury, 1997). An almost complete collection of gene deletion mutants of *S. cerevisiae* is available (Winzeler et al., 1999). These collections permit a comprehensive genome-wide view of drug action. New genomic technologies have been implemented in the yeast model organism: functional profiling (Giaever et al., 2002), gene expression profiling of drug action (Marton et al., 1998) or synthetic lethal screens (Tong et al., 2004). In a functional profiling study in yeast, the mode of action of 78 compounds was analysed and a novel target for the antitumor agent 5-fluorouracil was uncovered (Lum et al., 2004).

This new function has been confirmed by conventional molecular biological methods (Fang et al., 2004), providing proof of this principle for analyzing the modes of action of clinically relevant compounds. In a more recent study, more than 400 chemical compounds and diverse environmental stresses (Hillenmeyer et al., 2008)

The yeast gene deletion library was screened for sensitive or resistant growth phenotype in presence of cordycepin to gain further insight into the molecular effects of this drug and to identify new factors that are involved in RNA metabolism. The screening was performed by two different approaches. The first screen was a simple drop test, in which diluted yeast cultures were spotting on plates and growth of each yeast strain in presence and absence of cordycepin was analysed. As a second approach functional profiling was performed. This method allows the culturing of all yeast gene deletion strains in a competitive environment and relative fitness of each strain is visualised by microarray analysis. A big advantage of screening by functional profiling is its comprehensive genome-wide view of drug action and its fast and cheap screening procedure. This method is very sensitive and the relative fitness of each strain is represented quantitatively. Approximately 350 gene deletion strains are lost in this procedure because they are overgrown due to their extremely slow growth. A drop test screen is much more elaborate and is less sensitive, since growth of the mutant strains is compared by eye. Interestingly, our results revealed that the screening procedure and the culturing condition seemed to have a great impact on the outcome of the screen as the overlap of the two screens was surprisingly small (8.9%). Large-scale chemical genomic assays were usually carried out by functional profiling analysis (Lum et al., 2004; Parsons et al., 2006; Hillenmeyer et al., 2008). However, their result might be biased since possible targets might be only recognisable by a different screening approach like for example a drop test.

Different classes of cordycepin targets have been identified. One class included genes involved in CoTP synthesis. The *ADO1* deletion strain revealed a resistance against the drug (Iwashima et al., 1992). Ado1p converts adenosine into adenosine monophosphate (Lecoq et al., 2001) and is therefore necessary to convert the pro-drug cordycepin into the toxic compound CoTP. The resistance of the *ΔADO1* strain demonstrates that this conversion is absolutely essential for the mode of action of cordycepin. *ADK1* converts CoMP to CoTP (Konrad et al., 1988) and its deletion strain was also cordycepin resistant, although the cell contains other enzymes with a adenylate kinase activity (Cooper and Friedberg, 1992).

Furthermore, genes that may change ATP/CoTP ratio were identified. The absence of genes associated with peroxisome biogenesis (PEX genes) resulted in cordycepin sensitivity. Peroxisomes are small organelles with diverse functions: α- and β-oxidation of fatty acids, biosynthesis of ether phospholipids, oxidation and in mammals they are implicated as well in purine catabolism (Wanders and Waterham, 2006). Although it has not been demonstrated, that yeast peroxisomes are implicated in purine catabolism, we hypothesise that cordycepin might as well be degraded in peroxisomes. Disrupting the organelle structure and integrity might decrease the degradation efficiency resulting in a higher cordycepin concentration in the cell. Deletion strains of genes which encode for mitochondrial proteins were mostly only moderately sensitive to cordycepin. The core activity of mitochondria is the supply of energy by ATP synthesis. Deleting genes involved in ATP synthesis might result in a less efficient ATP synthesis changing the cellular ATP/CoTP ratio. The increased probability for an enzyme to interact with CoTP instead of ATP might lead to the observed growth inhibition of the respective deletion strain in presence of cordycepin.

In the group of genes whose deletion causes a resistant growth in presence of cordycepin, genes involved in mRNA turnover and ribosome biogenesis and translation were identified. Stabilising mRNAs by inhibiting degradation suppresses the toxicity of cordycepin and the respective gene deletion strains were resistant to the adenosine analogue even at much higher concentrations. The fact that mRNA stabilisation leads to cordycepin resistance provides further evidence that reduced RNA synthesis is the primary reason for cordycepin toxicity.

Interestingly, gene deletion strains of subunits of the SWR1 chromatin remodelling complex exhibited strong sensitivity to cordycepin. This complex deposits the histone H2A variant Htz1p into chromatin (Krogan et al., 2003). Consistently, the growth of *ΔHTZ1* was also highly inhibited by cordycepin. Chromatin remodelling occurs through two different classes of molecular processes. The first class remodels chromatin by post-translational modification of the protruding histones tails, such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation (Rando et al., 2003). The second class is ATP-dependent and is catalyzed by chromatin remodelling complexes. By changing the DNA–histone interactions ATPases can slide, eject, insert or restructure histone octamers (Saha et al., 2006). Htz1p has been shown to have a destabilizing effect on intranucleosomal interactions and as well on internucleosomal interactions (Abbott et al.,

2001). Genome-wide studies have demonstrated that Htz1p deposition happens at the vast majority at promoter regions (Guillemette et al., 2005). Destabilised nucleosome complexes could facilitate the binding of the RNA polymerase complex to the DNA. Such a function of Htz1p would be consistent with its primary location at promoter regions. *HTZ1* is not an essential gene, and *ΔHTZ1* does not show a general transcription defect (Guillemette et al., 2005). However, pull-down experiments demonstrated an association of Htz1p and RNA Pol II (Guillemette et al., 2005). It has been shown that the histone H2A analogue is required for full activation of *GAL1* and *GAL10* genes (Guillemette et al., 2005). Moreover, the cell cycle genes *CLN2* and *CLB5* require Htz1p at their promoter for their timely and complete transcriptional activation (Meneghini et al., 2003).

Most interestingly, we found in *ΔSWR1* and *ΔHTZ1* strains that the level and the length of the CUT *NEL025c* was increased in presence of cordycepin. This could mean that these two mutant strains either have an increased transcription rate of CUTs or that CUTs are more stable in these strains. Since Htz1p has been linked to promoters of actively transcribed genes (Millar et al., 2006) is unlikely, that these mutant strains have elevated CUT transcription rates. Htz1p incorporation into promoter regions facilitates transcription from the promoter, but might also hinder transcription of the respective CUT. Such a mechanism might explain our observation of increased *NEL025c* levels in *ΔSWR1* and *ΔHTZ1* strains. A provocative idea would be that Htz1p might be involved in the recruitment of the TRAMP complex and the exosome for efficient CUT degradation. A lack of Htz1p incorporation in the nucleosome might have a mild stabilising effect on CUTs, which can be enhanced by cordycepin.

Since we were mostly interested in new factors implicated at the interface of transcription and pre-mRNA processing, the further functional analysis was focussed mostly on genes coding for proteins with a nuclear localisation. Several candidates were further tested by a synthetic lethality test with *rna14-1*. From 23 tested genetic interactions, eleven were synthetic lethal, nine generated doublemutants had a severe growth defect and only three genes did not exhibit a genetic interaction with *RNA14*. The effect of cordycepin could be duplicated by mutation in a 3' end factor, which further underscores that genes identified in our cordycepin screening might indeed function in 3' end processing. Northern blot analysis revealed that mRNA levels of *ACT1* were affected in several mutant strains. Poly(A) tail length distribution analysis of most of those gene deletion strains showed that almost half of the examined strains showed an altered poly(A) tail distribution.

However, little is known about cellular pathways regulating poly(A) tail length. Many gene deletion strains exhibited an accumulation of short poly(A) tails indicating that the respective deleted gene might be implicated in 3' end processing. Analysis of termination by transcriptional run-on experiments was carried out with 15 cordycepin sensitive gene deletion strains. All 15 genes showed genetic interaction with *RNA14*, implicating that they might have a role in 3' end formation. However, none of the tested strains showed a defect in transcriptional termination that could be visualised by run-on technique. Further characterisation of the identified mutants may provide new insight into the mechanism of RNA synthesis.

Polyphosphate as a potential inhibitor of Pap1p activity

Most interestingly, our work identified poly P as potent inhibitor of Pap1p. Poly P is a chain of tens or many hundreds of phosphate residues linked by high-energy phosphoanhydride bonds and it is present in all species tested to date, from each of the three kingdoms of life (Kornberg et al., 1999). Poly P has numerous and varied biological functions depending on its location (species, cell, or subcellular compartment): substitution for ATP in kinase reactions, reservoir of P_i, chelation of metals (e.g. Mn^{2+}, Mg^{2+}, Ca^{2+}), buffer against alkali, mRNA processing and degradation, environmental remediation of phosphate, and regulatory roles in the physiological adjustments to growth, development, stress, and deprivation (Kornberg et al., 1999). It promotes ribosomal protein degradation by binding and activating the Lon protease in *E. coli* delivering amino acids needed to respond to starvation (Kuroda et al., 2001). In mammalian cells, the TOR kinase is activated by poly P under conditions of nutritional starvation (Wang et al., 2003).

We found that in *ΔPHO80* and *ΔPHO85* mutant strain polyphosphate accumulated and co-purified with the RNA. In the poly(A) tail labelling assay, polyphosphate inhibited the activity of Pap1p *in vitro*. A possible inhibitory connection of polyphosphate on poly(A) polymerase *in vivo* was indicated by the synergistic effect of the accumulated polyphosphate and cordycepin on Pap1p, which might be the reason for the strong sensitivity of these deletion strains to this drug. The observed synthetic lethality of *ΔPHO80* with *RNA14* underscored the hypothesis that polyphosphate might have a role in regulating Pap1p *in vivo*. We showed that polyphosphate bound Pap1p directly and we assume that it interacts with the DNA binding domain of Pap1p since it has a high

chemical similarity to the backbone of the DNA molecule. In yeast, cellular poly P levels can reach a concentration of 120 mM, but the majority is localised to the vacuole, but it has also been detected in the nucleus (Kornberg et al., 1999; Indge, 1986). Poly P levels in yeast are strongly dependent on growth phase and accumulate during diauxic shift induced by glucose depletion from the media (Werner et al., 2005). During this process, a complete rearrangement of gene expression takes place (Radonjic et al., 2005). Downregulation of expressed genes might be a result of the inhibition of polyadenylation by poly P. However, poly P level measurements in the nucleus are difficult.

A different phosphate molecule has been associated with the regulation of the recently identified Star-PAP in mammalian cells (Mellman et al., 2008). The activity of Star-PAP is highly increased in presence of phosphatidylinositol-4,5-biphosphate (Ptdlns-4,5P_2). Star-Pap has been shown to interact with the Ptdlns-4,5P_2 producing enzyme PIPKI, which allows the speculation that the associated PIPKI regulates the activity of Star-PAP by Ptdlns-4,5P_2 production. Polyphosphate might control the activity of yeast Pap1p in a similar mechanism, although the enzyme polyphosphate kinase has not been identified in yeast.

Mutation in *PAP1* triggers ATP synthesis

Paradoxically, mutations within *PAP1* caused resistance towards cordycepin. Several alleles were tested and all revealed cordycepin resistance and suppression of the extended RNA transcripts. This effect was unique to *PAP1* mutants. *PAP1* mutation also suppressed cordycepin toxicity in cordycepin sensitive mutants and doublemutants had no detectable poly(A) site shift. Gene expression profile of *pap1-1* revealed two important findings: Firstly, the process of ATP synthesis is upregulated in this mutant compared to wild-type strain. Secondly, cordycepin had little effect on the gene expression pattern in *pap1-1*, indicating that cordycepin is not effective in this mutant. Since CoTP competes with ATP in enzymatic reactions, we conclude that the ratio ATP/CoTP determines cordycepin toxicity.

Another interesting question arising from this observation is why ATP synthesis is induced in strains carrying a mutation in *PAP1*. Pap1p requires ATP for the synthesis of poly(A) tails. One possibility could be that Pap1p acts as a sensor for ATP levels in the cells. A change in Pap1p processivity by a mutation in *PAP1* could resemble a low ATP level situation that triggers ATP synthesis. Further experiments will be needed to answer

the mechanism of the proposed ATP synthesis activation signal. Fip1p is the only interaction partner of Pap1p in the PF I complex and has been shown to regulate Pap1p activity (Helmling et al., 2001). Fip1p is an essential protein and it plays a central role in regulating polyadenylation. Fip1p might be a candidate being implicated in the ATP synthesis signal mechanism.

Final remarks

Cordycepin is a nucleoside analogue has been implicated in various activities in mammalian cells. The activity against the parasites *Leishmania* and *Trypanosoma* (Berens et al., 1995; Rottenberg et al., 2005) but as well the anti-fungal activity against *C. albicans* (Eggimann et al., 2003) appear to be promising for a possible use of cordycepin for the treatment of these diseases. Since understanding the mechanism of the mode of action of a drug is essential for its therapeutic usage, the results presented in this thesis open new views on the molecular effects of cordycepin. Furthermore, our analysis may represent new starting points for the characterisation of genes involved in RNA synthesis.

VIII Material and Methods

4 Growth and handling of *S. cerevisiae*

Wild-type BY4741 (*Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and isogenic mutant strains were obtained from EUROSCARF (Winzeler et al., 1999). Other used strains are listed in table 4.1. Doublemutants were generated by disrupting the open reading frame of the respective gene with the *kanMX* or *NatR* cassette by homologous recombination in *rna14-1* (containing *RNA14* on a *URA3* plasmid) or *pap1-2* (containing *PAP1* on a *URA3* plasmid). Yeast strains were cultured in either full-medium or synthetic complete (SC) medium. Full-medium contained 1% yeast extract (Brunschwig), 2% Bacto-Peptone (Brunschwig) and 2% glucose (YPD). Liquid synthetic complete media contained 0.67% yeast nitrogen base (MP Biomedicals), 0.06% complete synthetic mix (lacking histidine, uracil or tryptophan) and either 2% glucose (SD) or 2% galactose (SGal). Plates contained 10% agar (Brunschwig). SD was supplemented with cordycepin purchased from Sigma at the indicated concentration or 5' fluoroorotic acid (1 mg/ml) from Zymo Research (Orange, CA, USA).

strain	genotype	reference
kin28-ts3	*Mat a*; *leu2*; *trp1*; *ura3*; *ade2*; *ade3*; *lys2*; *kin28-ts3*	Valay et al., 1995
pap1-1	*Mat a*; *ade1/ade2*; *lys2*; *gal?* ; *Ura3-52*; *pap1-1*	Patel and Butler, 1992
pap1-2	*Mat α*; *pap1::LEU2*; *pA-pap1-2-TRP1*; *ura3-1*; *his3-11*	Walter Keller lab
pap1-5	*Mat α*; *pap1::LEU2*; *pA-pap1-5-TRP1*; *ura3-1*; *his3-11*	Walter Keller lab
pap1-7	*Mat α*; *pap1::LEU2*; *pA-pap1-7-TRP1*; *ura3-1*; *his3-11*	Walter Keller lab
rat1-1	*Mat a*; *ura3-52*; *leu2Δ1*; *hisΔ200*; *rat1-1*	Amberg et al., 1992
rna14-1	*Mat a*; *ura3-1*; *trp1-1*; *ade2-1*; *leu2-3*; *112*; *his3-11*; *15*; *rna14-1*	Minvielle-Sebastia et al., 1994
rna15-1	*Mat α*; *ura3-1*; *trp1-1*; *ade2-1*; *leu2-3*; *112*; *his3-11*; *15*; *rna15-1*	Walter Keller lab
rpb1-1	*Mat a*; *ura3-52*; *rpb1-1*	Nonet et al., 1987
S288C	*Mat a*; *SUC2; gal2; mal; mel; flo1; flo8-1; hap1; ho; bio1; bio6*	Mortimer and Johnston, 1986
ssu72-2	*Mat a*; *ura3-1*; *trp1-1*; *ade2-1*; *leu2-3*; *112*; *his3-11*; *15*; *ssu72-2*	Walter Keller lab
ysh1-3	*Mat a*; *ura3-1*; *trp1-1*; *ade2-1*; *leu2-3*; *112*; *his3-11*; *15*; *TRP1::ysh1 [ysh1-3-HIS3-CEN]*	Walter Keller lab

Table 4.1 Strains used in this study.

5 Primers

primer	5' → 3' sequence	purpose
KanB rev	CTGCAGCGAGGAGCCGTAAT	strain verification
KanC forw	TGATTTTGATGACGAGCGTAAT	strain verification
NatR rev	GTGAAGGACCCATCCAGTGCCT	strain verification
Akr1 A forw	CGTTATCATTGTTAGAACTTTGGCT	strain verification
Apq12 A forw	GCCACAGACAGCTATCTCTATGAAT	strain verification
Apq12 B rev	GGAATCTTTTGACCAGACTAATGAA	strain verification
Bub1 A forw	CGTTTGACTTCACGTCCATGG	strain verification
Cst6 A forw	CAATGACAATGACAATGAAGGC	strain verification
Ecm30 A forw	CTTCAGGGGGTTTTTCCTTGCT	strain verification
Gtr1 A forw	CCGAGTCGTTTGAAGTCATCTC	strain verification
Htz1 A forw	GCTCCGTGCACGAAAACAACAA	strain verification
Isr1 A forw	GTAATATGCCCATCAAAACTCATC	strain verification
Mud2 A forw	TTATTTGCACTAATAATGCCAG	strain verification
Mud2 B rev	ATTCTCCCTGTTATATGATCTTCCC	strain verification
Pep5 A forw	CAGGATGTGTTACGATGCAGACA	strain verification
Pho2 A forw	TATGGAGTTAAAGTGTGGGATTGT	strain verification
Pho4 A forw	TACGGTGTGTATGTGTATGTCTGTG	strain verification
Pho4 B rev	CGCTTGTTCAAATACGGACTACTAT	strain verification
Pho5 B rev	ATAGTCGCCAGGGAAAGAGTAGTAT	strain verification
Pho80 A forw	AAACAATCAGTCTCCATACTCATCC	strain verification
Pho80 B rev	TCTCCAGTATATTCAATTCGTGACA	strain verification
Pho81 A forw	AGTTTTTGAGAGTCCCTTCAAGTTT	strain verification
Pho81 B rev	GGAAACAACAGTAGCAAGGTAAAAA	strain verification
Pho84 A forw	GCGTATTACTCATTAATTAACCGACC	strain verification
Pho84 B rev	TTGACCAATAACAGTACCAACAGAA	strain verification
Pho85 A forw	AATGAAATTTTTAACAATGCAGAGC	strain verification
Pho85 B rev	AGTTTAGTTATAGCCCTCTTGGTGT	strain verification
Pho86 A forw	CAATAATGGAAATCCAGATGGTG	strain verification
Pho86 B rev	TTATCGGACTTTCTACCCTCTTTCT	strain verification
Pho87 A forw	AAGGTTTAAGATTGTGCATAGGAAA	strain verification

primer	5' → 3' sequence	purpose
Pho87 B rev	ATCATCTACTTTCAATTTCTCGTCG	strain verification
Rai1 A forw	CTCAGCTGCTTGTATAGCCTA	strain verification
Rai1 B rev	ATTTCCGTGCTATTCCTCTAAAAGT	strain verification
Rmd7 A forw	GGGGATGAGATGGTTGCTCTT	strain verification
Rpb9 A forw	CGGGCTGCTTGTCCTGTTCCTT	strain verification
Sac1 A forw	TTTAGTAACCTGTAGACCTTCA	strain verification
Sac3 A forw	CTTTATCCACTAGCCCTTTTTCTTC	strain verification
Sit4 A forw	GAGCATTGAAGAGCTACAGACG	strain verification
Snf1 A forw	GGCCAAGACATAGCTTTGGGCTT	strain verification
Snf2 A forw	CAAATTGATTGACGAAACGGGG	strain verification
Snf6 A forw	GCGGCTGGACCATACAGGTTCT	strain verification
Spt8 A forw	CGGCTATGAAAAAAATGGCAGAG	strain verification
Spt8 B rev	GAATGCTTCTGCAGGATAGTTAGAG	strain verification
Swr1 A forw	TTGAAGAAAGAGTGCAAAGGGA	strain verification
Thp1 A forw	CTGGAATATGAGCAGACAATAGTAT	strain verification
Thp1 B ref	TATCAACATTTCTATGTGAGGAGCA	strain verification
Vtc4 A forw	ACGTAACCCATCATAGGAAATTGTA	strain verification
Vtc4 B rev	TGCAAAATGTGTAAACCTTGTCTAA	strain verification
YDL172c A forw	GTGGTGACTGTAGAAGAGGGCAAT	strain verification
YDL172c B rev	ACATAATTTCTCCAAAGTCAAGCAG	strain verification
YDL173w A forw	GGCTCCAAGAGACAGCGACCGT	strain verification
YDL173w B rev	GTTTGCTACTGTCTTTGAATTGGAT	strain verification
Yke2 A forw	CTGTAGTGCGATGATATCTTGGT	strain verification
Akr1 150 bp forw	CCCTCAATATGAACGATCTATA	*kanMX* gene deletion cassette
Akr1 150 bp rev	CCACCTCATGAGCTTTCCTAAT	*kanMX* gene deletion cassette
Apq12 150 bp forw	TGTTCTTGTCTTTCTCTCTCCT	*kanMX* gene deletion cassette
Apq12 150 bp rev	AAATCAAGTCACAATTGTAATG	*kanMX* gene deletion cassette
Bub1 150 bp forw	CGATACGCGCTAGGAAGTAAA	*kanMX* gene deletion cassette
Bub1 150 bp rev	CCATAAGTGACAGATGTCAAGA	*kanMX* gene deletion cassette
Cst6 150 bp forw	GAGGTTATCGTGTATACGTATC	*kanMX* gene deletion cassette
Cst6 150 bp rev	CGGCAGAAAAGAAAGGATGCAG	*kanMX* gene deletion cassette

primer	5' → 3' sequence	purpose
Ecm30 150 bp forw	GGTGTTTTTTCTTTCGGGTAAA	*kanMX* gene deletion cassette
Ecm30 150 bp ref	GAAGATTTTTACTTTTCTTCAA	*kanMX* gene deletion cassette
Gtr1 150 bp forw	GCCTCTTCTTTATCACTCTCGC	*kanMX* gene deletion cassette
Gtr1 150 bp rev	GCTTTTTCCTCCCATTGTCCTAT	*kanMX* gene deletion cassette
Htz1 150 bp forw	CAGCTCTATTTAACAGCTGAAC	*kanMX* gene deletion cassette
Htz1 150 bp rev	ACGGCTACTGCGCTATCGTT	*kanMX* gene deletion cassette
Isr1 150 bp forw	GCGCTGCTAATAGATTCTTGCCT	*kanMX* gene deletion cassette
Isr1 150 bp rev	GGATGAAGAAAAGGAAAGGCG	*kanMX* gene deletion cassette
Mud2 150 bp forw	TACGCTGTAGAATGGATCACTT	*kanMX* gene deletion cassette
Mud2 150 bp ref	TTTTCCAGATCGCAAGTTAGTA	*kanMX* gene deletion cassette
Pep5 150 bp forw	GTGGTGTACGGATTATGTGTAACTG	*kanMX* gene deletion cassette
Pep5 150 bp rev	CGGTTGCCACATTAATAATTTT	*kanMX* gene deletion cassette
Pho4 150 bp forw	AAGTGCGATTTTCTCGTTTTCT	*kanMX* gene deletion cassette
Pho4 150 bp rev	CTTCTGTTAATGTGAGTGCGTGT	*kanMX* gene deletion cassette
Rai1 150 bp forw	GAGTAAGCATTCGGGTAAATTG	*kanMX* gene deletion cassette
Rai1 150 bp rev	CGAGTTTCGGATCATACAGGG	*kanMX* gene deletion cassette
Rmd7 150 bp forw	GCGCGTTTGCTGGGACAAAAGA	*kanMX* gene deletion cassette
Rmd7 150 bp rev	CTCCAATTCAGTTCTATACATGC	*kanMX* gene deletion cassette
Rpb9 150 bp forw	TTAGTTTGAGTTGTTTTATTGG	*kanMX* gene deletion cassette
Rpb9 150 bp ref	CGGTGCTAAGATCAGGATTTTT	*kanMX* gene deletion cassette
Sac1 150 bp forw	TGCTGCACTACTGCTTACCCAC	*kanMX* gene deletion cassette
Sac1 150 bp rev	CATGAAAGATAAAGTAAAAAAG	*kanMX* gene deletion cassette
Sac3 150 bp forw	CAGAGAGCGTAGTGATGCATA	*kanMX* gene deletion cassette
Sac3 150 bp rev	CCCTGGCTTCACTTTTTGCACA	*kanMX* gene deletion cassette
Sit4 150 bp forw	AATAGGTTATTGCATACTATCA	*kanMX* gene deletion cassette
Sit4 150 bp rev	AATTTTTTATTTGGTCATTTTG	*kanMX* gene deletion cassette

primer	5' → 3' sequence	purpose
Snf1 150 bp forw	CCAAACAGTCATTCAGGAAGTA	*kanMX* gene deletion cassette
Snf1 150 bp rev	CCGAAGAAATAATGCCAATAAAA	*kanMX* gene deletion cassette
Snf2 150 bp forw	GTGACGTACGTGGACCTTTTGT	*kanMX* gene deletion cassette
Snf2 150 bp rev	GATATTAAACATCCCAACTCGG	*kanMX* gene deletion cassette
Snf6 150 bp forw	GGAAAAGATGGGAGAGAAAAAA	*kanMX* gene deletion cassette
Snf6 150 bp rev	GCTGCACAAATCCACTATGTCA	*kanMX* gene deletion cassette
Spt8 150 bp forw	CTTCCAGAATGCCCTTTCAGGA	*kanMX* gene deletion cassette
Spt8 150 bp rev	GCCTTTGTCTGCCCGGACCCTG	*kanMX* gene deletion cassette
Swr1 150 bp forw	AAAAGGATAGATTTTGAGCTTT	*kanMX* gene deletion cassette
Swr1 150 bp rev	AACAATGCCATGGTGAGTACAT	*kanMX* gene deletion cassette
Thp1 150 bp forw	GTCGTTGACTTTCTCTCCCTTT	*kanMX* gene deletion cassette
Thp1 150 bp rev	TGTTACTCCCTTTTTTCCTCCT	*kanMX* gene deletion cassette
YDL172c 150 bp forw	GGTGGTATCGTTCAACGTGATT	*kanMX* gene deletion cassette
YDL172c 150 bp rev	CGTTTTGCAATTGGTAGAGGT	*kanMX* gene deletion cassette
YDL173w 150 bp forw	CCAGACGAGTTCCTTTATAACGT	*kanMX* gene deletion cassette
YDL173w 150 bp rev	GGTGGTATCGTTCAACGTGATT	*kanMX* gene deletion cassette
Yke 2 150 bp forw	GAAGTCTGAAATTTTGATGCGA	*kanMX* gene deletion cassette
Yke 2 150 bp rev	TTTAGGACAGTGAAATGCGTAGG	*kanMX* gene deletion cassette
ΔPho80-NatR forw	TCTGCAAGCTATCATAAGACGAGGATA TCCTTTGGAGACTCATAGAAATCCACT ACGATTTAGGTGACAC	*NatR* gene deletion cassette
ΔPho80-NatR rev	TTTAATTTTGCTCAATCATGATTGCTTT CATAATACCCCACGAAAAATCAAATAC GACTCACTATAGGGAG	*NatR* gene deletion cassette
Cyh2 forw	AGCACAGAGGTCACGTCTCA	PCR probe for hybridisation
Cyh2 ref	ATTCAACAACACCACCAGCA	PCR probe for hybridisation
Cyh2 intron forw	ATGTAGTTCCATTTGGAAGAGG	PCR probe for hybridisation
Cyh2 intron ref	AACAAAAGAAACGTGGTAACTC	PCR probe for hybridisation
Act1 forw	TCTTCCCATCTATCGTCGGT	PCR probe for hybridisation
Act1 ref	AGTTTGGTCAATACCGGCAG	PCR probe for hybridisation

primer	5' → 3' sequence	purpose
Asc1 forw	TTGACTGGTGACGACCAAAA	PCR probe for hybridisation
Asc1 rev	AGCAGACCAAGCCAAAGAAA	PCR probe for hybridisation
Adh1 forw	GTCACACTGACTTGCACGC	PCR probe for hybridisation
Adh1 rev	TGTCAGCTCTGTTACCGACG	PCR probe for hybridisation
Pgk1 forw	TCCCATTGGACGGTAAGAAG	PCR probe for hybridisation
Pgk1 rev	GTGTTGGCATCAGCAGAGAA	PCR probe for hybridisation
Nel025 forw	CTGTTGACATTGCAGACAAT	PCR probe for hybridisation
Nel025 rev	GTGGTTAGAAGATTAATGTGAT	PCR probe for hybridisation
U3/snR17a forw	AGGACATTTCTATAGGAATCG	PCR probe for hybridisation
U3/snR17a ref	AAAGAAGTACATAGGATGGGTC	PCR probe for hybridisation
$tRNA_3^{LEU}$	GAACTCTTGCATCTTACGATAFC	hybridisation
18S rRNA	ACGAAAAATCAAATAC	hybridisation
Act1 RNase H	ACATAACGATGTTACCGTATAATTC	RNase H digest of *ACT1*
Asc1 RNase H	AAGAGAAAACTTCATCTTGGGCAGA	RNase H digest of *ASC1*

Table 4.2 Primers that were used in this study.

6 Drop test

The strains were grown over night in 200 µl of the respective media and temperature and three ten-fold serial dilutions were made. 4 µl of each dilution were spotted on different yeast plates and incubated for 3 days at the respective temperature.

7 Growth curve

The wild-type strain BY4741 was cultured in SD media at 30°C in a water bath overnight and diluted in the morning to an OD_{600} of 0.05 (50 ml cultures) in pre-warmed cordycepin containing (0, 10, 20, 40 µg/ml) SD media and incubated again at 30°C. The OD_{600} was measured every hour and cultures were diluted to an OD_{600} of 0.05 when they reached an OD_{600} of 0.4 - 0.5. The OD_{600} was graphically displayed against the time. The percentage of inhibition was calculated with the added trend line equation.

8 Functional profiling

43 cell pools obtained from Alain Jacquier (Institute Pasteur, Paris) were pooled and mixed. 100 ml SD was inoculated by the cell pool and this pre-culture was incubated overnight at 30°C in a water bath. When the pre-culture reached an OD_{600} of 1, the culture was diluted to an OD_{600} of 0.05 in SD and SD containing 20 µg/ml cordycepin in duplicate. The OD_{600} was measured every hour and growth was monitored in a graph. After 12 and 18 generations, 10 ml of cell culture was harvested by centrifugation (5 minutes, 3500 rpm, 4°C) and shortly washed with ice-cold water. The cell pellet was frozen in liquid nitrogen and stored at - 80°C. Genomic DNA extraction was performed as described below. Labelling of the probes, hybridisation to arrays and analysis of the data was done by Florence Decourty and Alain Jacquier in Paris (Decourty et al., 2008).

9 Transformation of yeast cells

Yeast transformation was carried out as described by Schiestl and Gietz (1989). 50 ml of yeast were grown in YPD to an $OD_{600}$of 0.5 to 0.8 or in SD to an OD_{600} of 0.3 to 0.5 and harvested by centrifugation for 3 min at 3600 rpm. After washing with 10 ml H_2O, the pellet was resuspended in 25 ml transformation solution (10 mM Tris-HCl pH 7.5, 100 mM LiOAc, 10 mM DTT) and incubated at room temperature for 40 minutes with gentle shaking. The cells were centrifuged for 3 minutes and resuspended in 750 µl of transformation solution. 5 µl Mini-Prep DNA or 1 - 2 µg purified PCR product was provided in a tube, with 5 µl carrier DNA (DNA of salmon or herring testis, 10 mg/ml), 50 µl LiT (10 mM Tris-HCl pH 7.5, 100 mM LiOAc) and 100 µl of cells was added and the mixture was incubated for 10 minutes at room temperature. 300 µl PEG solution (10 mM Tris-HCl pH 7.5, 100 mM LiOAc, 40% PEG-4000) was added and the cells were incubated for 10 minutes at room temperature. After the addition of 50 µl DMSO, the transformants were heat-shocked for 15 minutes at 42°C. The cells were centrifuged, resuspended in 1 ml of YPD and incubated for 60 to 120 minutes at 25°C or 30°C. After the incubation, the cells were centrifuged and washed with 1 ml of H_2O. The pellet was resupended in 50 µl H_2O and plated on selective medium. For plasmid transformations the cells were washed and plated immediately after the heat shock. To transform yeast cells

grown on plate, one loop of freshly restreaked yeast cells was washed twice in 1 ml of H_2O and once with 1 ml LiT. The pellet was then resuspended in 25 ml transformation solution and incubated for 40 minutes at room temperature. All further steps were performed as described above.

10 Preparation of genomic DNA

10 ml of an overnight yeast culture was centrifuged (3 min, 3500 rpm) and washed with 10 ml H_2O. The cells were resuspended in 200 µl extraction buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). 200 µl glass beads and 200 µl phenol:chloroform:isoamylalcohol (25:24:1) were added and the mixture was vortexed for 7 minutes. 200 µl of extraction buffer was added and shortly vortexed. After a centrifugation for 15 minutes at 15'000 rpm and 4°C the upper phase was removed and extracted with an equal volume of chloroform. Genomic DNA was precipitated using 1 ml of 100% ethanol and incubation of the solution for 10 min at -20°C. After a centrifugation for 15 min at 4°C and 16'000 rpm, the pellet was washed with 300 µl of 70% ethanol. The pellet was dried and resuspended in 30 µl TE/RNase A (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 10 ng/ml RNase A) and stored at -20°C.

11 RNA extraction

RNA was extracted from yeast cells according to a hot phenol procedure essentially as described before (Collart and Oliviero, 2001). When not indicated differently, the cells were grown in YPD or SD media to an OD_{600} of 0.8 and harvested by centrifugation for 5 minutes at 3500 rpm. The cell pellet was washed with 10 ml ice-cold H_2O. Cell pellets were resuspended in 600 µl TES (10 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0, 0.5% SDS). After the addition of 500 µl acid phenol (pH 5, Roth), the cells were incubated at 65°C and vortexed three times for 15 seconds, followed by an incubation time of 15 minutes. This procedure was repeated at least four times. The samples were centrifuged for 5 minutes at 15'000 rpm at 4°C. The upper phase was transferred to 500 µl phenol and

extracted by vortexing three times for 15 seconds. After centrifugation the upper phase was transferred to 500 µl chloroform. The samples were vortexed again three times for 15 seconds and centrifuged at 4°C. RNA was precipitated by the addition of 35 µl 3 M NaOAc and 1 ml 100% ethanol following the incubation for at least 2 hours at -20°C or 15 minutes at -80°C. The RNA was pelleted by centrifugation for 20 minutes at 15'000 rpm and 4°C. The pellet was washed with 500 µl 70% ethanol and dried in a speedvac for 3 minutes. The RNA pellet was dissolved in 100 µl H_2O_{DEPC} (0.1% DEPC) and the RNA concentration was measured using the NanoDrop (Thermo Scientific).

12 Northern blotting

15 µg of RNA was mixed with 20 µl formaldehyde loading buffer (50% formamid, 6% formaldehyde, 0.4 M HEPES, 0.025% xylene cyanol FF, 0.025% bromophenol blue, 15% glycerol) and denatured for 5 minutes at 65°C. The RNA was loaded on a 1.2% agarose gel (0.05 M HEPES pH 7.8, 1 mM EDTA) and 50 volts were applied for at least 16 hours. The gel was washed 10 minutes with H_2O, and then the RNA was partially hydrolysed in 75 mM NaOH for 20 minutes. The gel was neutralised by incubation in neutralising solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for 15 minutes. This step was repeated, then the gel was incubated in 10x SSC (1.5 M NaCl, 0.15 M sodium citrate pH 7.0) and the RNA was transferred overnight to a Hybond-N^+membrane (Amersham Biosciences) with 10x SSC. The RNA was UV cross linked and the membrane was wrapped with saran foil for storage.

For hybridisation, the membrane was stripped if necessary for 1 hour at 70°C in 0.1% SDS. For the hybridisation with a PCR probe, the membrane was pre-hybridised in pre-hybridisation buffer (50% formamid, 5x SSC, 5x Denhard's solution, 1% SDS, 0.2 mg/ml DNA of salmon or herring testis) in a Hybaid oven for 30 minutes at 65°C. The PCR probe was initially denatured for 3 minutes at 100°C and immediately put on ice. The probe was labelled with [α-^{32}P]-dATP using the random prime labelling kit (Roche). After an additional denaturation step, 900 µl of pre-hybridisation buffer were added and the probe was filtered (0.22 µm) and added to the membrane. The membrane was hybridised at 37°C over night. The membrane was washed with 6x SSPE, 1x SSPE 0.1% SDS, 0.2x SSPE 0.1% SDS and 0.1x SSPE 0.1% SDS for 30 minutes each at 42°C. For oligo

primer hybridisation the membrane was pre-hybridised in oligo-prehybridisation buffer (4x SSPE, 5x Denhard's solution, 0.5% SDS, 0.2 mg/ml DNA of salmon or herring testis). The probe was labelled with [γ-^{32}P]-ATP using the enzyme T4 polynucleotide kinase (Fermentas) and after an incubation time of 30 minutes at 37°C the labelled oligo probe was filtered to the membrane and hybridised overnight at 37°C. The membrane was washed with 6 x SSC for 15 minutes at 30°C, 37°C and 42°C each. After the washing, the membrane was exposed to a phosphor imager screen for at least 1 day and visualised on a FLA-7000 phosphor imager (Fuji).

13 Poly(A) length analysis

Assays were performed essentially as described (Martin and Keller, 1998). 2 µg of total RNA was incubated with 400 ng recombinantly expressed yeast poly(A) polymerase (a gift from G. Martin, Basel) and 0.2 µl [α-^{32}P]-cordycepin triphosphate (Perkin-Elmer) in reaction buffer (20 mM Tris-HCl pH 7.0, 50 mM KCl, 0.7 mM $MnCl_2$, 10% glycerol, 100 µg/ml BSA) for 30 minutes at 30°C in a total volume of 12 µl. After heat inactivation, RNA was digested with RNase A and RNase T1, followed by proteinase K treatment. Precipitated RNAs were resolved on 15%/8.3 M urea polyacrylamide gels, that were exposed and visualized on a FLA-7000 phosphor-imager (Fuji)

14 RNase H digestion

10 µg of total RNA was mixed with 2 µl of oligo primer (10 mM) and 4 µl H-buffer (100 mM KCl, 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTT). The RNA was denatured for 5 minutes at 75°C and the pimers were annealed to the RNA by incubating for 1 hour at 37°C. 1 µl RNase H (Invitrogen) together with 0.5 µl RNase Out (Invitrogen) and 0.5 µl H-buffer were added and the RNA was digested for 1 hour at 37°C. 25 µl H_2O_{DEPC} was added and the RNA was extracted with 50 µl phenol:chloroform: isoamylalcohol (25:24:1). The RNA was precipitated with ethanol and resuspended in 20 µl formamide loading buffer (98% formamide, 0.025% xylene cyanol FF, 0.025%

bromophenol blue, 10 mM EDTA pH 8.0). The RNA was separated on an 8%/8.3 M urea polyacrylamid gel for 8 hours at 300 V. The transfer to a Hybond-N^+ membrane (Amersham, Bioscience) was performed in 0.5x TBE overnight at 30 V and 200 mA using a wet transfer blotting system (Biorad). The membrane was UV cross-linked and the membrane was wrapped in foil for storage.

15 Isolation of M13 phages

100 ml of 2x YT medium was inoculated with a one colony of the *E. coli* strain J101 and incubated overnight at 30°C. In the next morning, the culture is diluted to an OD_{600} of 0.1 and incubated at 37°C until the culture reached an OD_{600} of 0.3 to 0.5. 50 µl of M13 phage containing *CYC1* probe 1 to 6 or Actin (Birse et al., 1998) was each added to 2 ml of the *E. coli* culture and incubated for 5 minutes at room temperature. These 2 ml pre-cultures were used to inoculate 200 ml of 2x YT (pre-warmed), which were incubated at 37°C four 5 hours. The cells were harvested by centrifugation for 15 minutes at 8000 rpm with a GS3 rotor. The supernatant was collected and phage particles were precipitated overnight in solution I (1.25 mM PEG-8000, 0.125 M NaCl) at 4°C. DNA was pelleted by centrifugation for 1 hour at 13'000 rpm at 4°C in a SLA rotor. The supernatant was removed and the centrifugation was repeated for 20 minutes, followed by a complete removal of the remaining supernatant. The pellets were air-dried for 5 minutes and resuspended in 500 µl TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). DNA was isolated by phenol extraction and precipitated by addition of ethanol and incubation for 15 minutes at -80°C. DNA was collected by centrifugation for 20 minutes at 15'000 rpm at 4°C and the pellet was washed with 500 µl 70% ethanol. The pellet was dried in the speedvac for 3 minutes and resuspended in 400 µl TE buffer. DNA concentration was measured with NanoDrop (Thermo Scientific).

16 Transcriptional run on (TRO)

TRO analysis was performed essentially as described by Birse et al. (1998). The yeast strains of interest were transformed with the plasmid pBD366 containing CUP-CYC1 or

pBD346 containing GAL1/10-CYC1. 50 ml cultures were grown in SD containing 0.2 mM $CuSO_4$ or SGal to an OD_{600} of 0.1 – 0.2 harvested by centrifugation for 5 minutes at 3500 rpm at 4°C. The cells were washed with 1 ml cold H_2O and resuspended in 0.5% sodium-lauryl-sarcosine sulphate. After 20 minutes incubation on ice, the cells were pelleted by centrifugation for 1 minute at 8'000 rpm at 4°C and the supernatant was removed carefully. The centrifugation step was repeated and the remaining liquid was removed completely with a pipette. The cells were resuspended in 60 µl TRO buffer (50 mM Tris-HCl pH 7.7, 500 mM KCl, 80 mM $MgCl_2$) in the presence of ATP, GTP and CTP (10 mM each) and 2 mM DTT. The transcription reaction was initiated by the addition of 3 µl [α-^{32}P]-UTP and allowed to proceed for 5 minutes at 30°C. The reaction was stopped by the addition of 900 µl AE buffer (50 mM NaOAc, 10 mM EDTA). Total RNA was then purified by hot phenol extraction (described above) and partially hydrolysed by incubation on ice for 5 minutes in the presence of 0.2 M NaOH and neutralised by the addition of 20 µl neutralising solution (0.5 M Tris, 0.5 M HCl). 6.7 µg of M13 phage DNA was applied on a Hybond-N^+membrane (Amersham, Bioscience) using a slot blot manifold (BioRad). DNA was denatured by incubating in denaturing solution (1.5 M NaCl, 0.5 M NaOH) and filters were neutralised in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) and UV cross linked. Hybridisation of the labelled RNA was performed overnight at 42°C in hybridisation buffer (50% formamid, 6x SSPE, 5x Denhard's solution, and 0.1% SDS). Filters were washed for 15 minutes each with 2x SSC, 0.1% SDS at 42°C, 0.2x SSC, 0.1% SDS at 30°C and 0.1x SSC, 0.1% SDS at 37°C and exposed and visualized on a FLA-7000 phosphor-imager (Fuji).

17 Cell growth and RNA extraction for microarray analysis

An overnight pre-culture of wild-type S228C and *pap1-1* were diluted to an OD_{600} of 0.05 in SD medium and incubated at 25 °C until the culture reached an OD_{600} of about 0.3. Cordycepin was added to a final concentration of 20 µg/ml and cells were allowed to grow for 1 hour. *Pap1-1* was additionally shifted to 37°C for 1 hour. All cultures were grown in triplicates. Cells were harvested by centrifugation and washed with cold H_2O. The pellet was frozen in liquid nitrogen and stored at -80°C. RNA was extracted according to the hot

phenol method as described above. To further purify the RNA, the RNeasy Micro Kit (Qiagen) was used.

18 Oligo array hybridisation and analysis of data

10 µg total RNA was mixed with oligo(dT) and pdN_9 primer (5 µg/µl each) and 1 µl of Ambion spike control. The RNA was denatured for 10 minutes at 70°C and chilled on ice for 10 minutes. Then, the RNA was reverse transcribed using superscript II (200 U/µl) (Invitrogen) and 0.6 µl of aa-NTP mix (25 mM dATP, 25 mM dGTP, 25 mM dCTP, 12.5 mM dTTP, 12.5 amino-allyl-dUTP (Sigma)) for at least 2 hours at 42°C. The RNA was hydrolysed by the addition of 10 µl 0.5 M NaOH and 10 µl 0.5 M EDTA pH 8.0 for 15 minutes at 65°C. The solution was neutralised with 25 µl 1 M HEPES pH 8.0. The DNA was cleaned from buffer remains and concentrated to a final volume of 9 µl using the Microcon-30 concentrator (Amicon). NHS-Cy3 and NHS-Cy5 dyes (Amersham) were resuspended in 10 µl DMSO. For the coupling reaction, 1 µl of the respective dye and 1 µl 1 M sodium bicarbonate pH 9.0 was added to the DNA was incubated for 1 hour at room temperature. Remaining NHS-dye was aliquoted and dried in the speed vac for around 2 hours and stored in a dessicator at 4°C. The labelled DNA was concentrated and purified using the Qia-quick PCR purification kit (Qiagen) by a final elution of 60 µl. The Cy3- and Cy5-labelled cDNA samples were mixed and concentrated in Microcon-30 concentrators (Amicon) to a final volume of 10.5 µl. Labelling efficiency was measured by NanoDrop using the microarray application.

Nexterion oligo arrays were post-processed in blocking solution (5x SSC, 4 mg/ml BSA, 0.1% SDS) inside of the waterbath for 60 minutes at 42°C. The arrays were washed for 10 minutes in 0.3x SSC for three times, shortly rinsed by water and dried by centrifugation for 2 minutes at 500 g. The concentrated labelled DNA was mixed with MWG formamide buffer (Ocimun Biosolutions) and poly(A) RNA was added to a final concentration of 0.5 mg/ml. The hybridisation mix was incubated at 80°C for 10 minutes and loaded on the array. A coverslip was added on top and the array was enclosed in a slide chamber and hybridised in a waterbath overnight at 42°C. The arrays were washed for 12 minutes each in 2x SSC, 0.2% SDS, 2x SSC and 0.2x SSC and immersed with 100% ethanol to fix the cDNA to the array. The arrays were dried by centrifugation and

scanned with Axon instruments scanner 4000 (Foster City, California, United States). Scanning parameters were adjusted to similar fluorescent intensities in both channels. Scanning data were collected with the software GENEPIX 3.0 (Axon Instruments) and spots with abnormal morphology were identified by eye and excluded from further analysis. Arrays were normalized by the Stanford Microarray Database (SMD) (Gollub et al., 2003). The calculated $\log_2$ ratios were imported into Acuity 4.0 (Axon Instruments) and filtered for regression correlation of greater than 0.5 (filters for large variations in the ratios of pixels within each spot) and a signal to noise ratio of greater than 2.5 (signal over background). Average $\log_2$ rations were calculated from the triple experiments for each gene spot. RNA were considered as increased when the $\log_2$ ratio was > 1 and decreased when $\log_2$ was < -1.

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